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ELECTRON STIMULATION OF BIOPOLYMERS

(Electron-stimulated States of Biopolymers)

by
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This monograph represents the first attempt, in the biophysics literature in this country and abroad, to compile and organize works, relative to the discovery and studies of the nature of electron-stimulated states of the two principal classes of biopolymers: proteins and nucleinic acids. Particular attention is given to the biological role of the electron-stimulated states, the mechanism of their appearance, migration and prolonged concentration of energy. Problems are reviewed in reciprocal spectral luminescence characteristics of biopolymers with their secondary, tertiary and quaternary structures, potential application of photoluminescence of the biopolymers, as a source of information on the physico-chemical and structural organization of the vital cells on the molecular and supramolecular levels.

Intended for biophysicists, biochemists, cytologists, radiobiologists and students of corresponding colleges and universities.

Introduction

Almost eight years have passed since the appearance of the book by Read "Stimulated Electron State in Chemistry and Biology" - which was a magnificent view in depth and width of the problem - During this period of time, there was continued further intensive accumulation of data on the nature and the role of the electron stimulated states in biology. These data was concentrated, first of all, around the electron stimulated states of pigmented systems, the normal biological functioning of which presumes reciprocal effect between molecules and the quanta of light.

spelling?: Reede, Reade ?

At the same time, during the last decade, there was actually laid the foundation to a vigorous research study on the electron stimulated states of biopolymers and, first of all, proteins and nucleinic acids. These research projects were carried out very extensively and included absorption, luminescence and photochemical methods, as well as the methods of spectral effect. Although, in contrast to various types of molecules in pigments, the reciprocal effect with the quanta of radiation energy is not included in the field of biological "duties" of biopolymers, in spite of the fact that their everyday functioning belongs with the type of most marked dark reactions, nevertheless research in this direction is of definite interest for biology, at least from three different points of view.

In the first place, both naturally and artificially, biopolymers have to undergo the effect of ultraviolet and ionizing radiation. In this connection, one might mention the entire

circle of problems in radiobiology, the effect of artificial and natural sources of penetrating radiation upon the organism; the effect on the microbe, vegetative and animal organisms, of the ultraviolet component of solar radiation; the application of artificial sources of ultraviolet light for the purposes of combat against harmful organisms (microbes), indended to stimulate new beneficial or to eliminate noxious hereditary signs of microorganisms in mutagenesis and radioselection, the application of artificial ultraviolet irradiation of animals in agriculture as therapy and stimulation of productivity. In all these cases, the chain of events (leading) to the change in biological properties or cellular activity and organisms, to a large degree, starts with the electron stimulated states of proteins and nucleinic acids, as the basic acceptors, receiving the effect of ultraviolet and penetrating radiation. As a matter of fact, any biological process may be transformed (inhibited, stimulated or altered), as a result of the photochemical route of realization (production) of electron stimulated states of proteins and nucleinic acids. It is obvious, in this connection, that knowledge of the nature natureof the electron stimulated states, photophysical processes in energy exchange and the primary photochemical reactions of biopolymers plays the leading role the understanding and conscious regulation of all these processes.

In the second place, studying the electron stimulated states with the aid of photoabsorption, =hotoluminescence and hemiluminescent methods, we thereby acquire a great volume of information,

not alone as to the quantity of molecules in biopolymers in the system, but likewise as to their qualitative condition. As we shall indicate below, in a number of cases, the luminescence quanta bring information even on the structural organization of macromolecules, and on their configuration. Taking into the Account the fact that such information may be obtained from the inside of the intact, normally functioning cell actually momentarily, then the importance of spectral luminescence methods of research in biology assumes particular significance.

Then, finally, in the third place, we may believe that the features of dark and light behavior of the molecules conceal a profound unity. Studying the details of electron stimulation of molecules, we thereby acquire information of the vast architecture of their energy levels, permissible and non permissible states of the olouds of external electrons of moleculres. At the same time, the same external electrons predispose biochemical and biophysical behAvior of the molecules also in the dark. The role of optical transitions in the dark, the origin of the electron stimulated states in the course of darkness metabolism bioluminescence and mitogenetic rays, as a means of physical co tact between the cells - all these problems touch upon one of the most exciting and at the same time the least of all solved problems in molecular biology.

HISTORY OF THE STUDY OF ELECTRON-STIMULATED STATES OF PROTEINS

During the last decade there was discovered and by now sufficiently studied in detail the new physical property of proteins - their luminescence.

In spite of its relative youth, the problem of protein luminescence possesses rather extensive preliminary history. Apparently, bAck in the 18th century, Bakhari (Soviet spelling) first stumbled unto the luminescence of tissue, rich in proteins, as he observed the luminescence of his own hand in the dark room, after a short period of exposure to sunlight. Stocks (spelling?) (quoted in Dhere, 1933, 1935) xxxobserved fluorescence of horns and nails. In 1858, Bngolt and in 1959 Sechenov observed fluorescence of the chrystalline lens and skin, and in 1883 Soret (spelling?) described violent fluorescence of myosin in 1^{o}/o hydrochloride. Helmholtz, 1896, observed fluorescence of the eyes of vertebrate animals, while Hess, 1911, that in insects and the crustacenas. Stubel in his work, 1911, described fluorescence of skin.

The third decade of our century ("the 30's"), marked by a fad for the black color (the source of artificial ultraviolet rays), brought about a number of qualitative observations on the fluorescence of most varied protein bodies. This luminescence became the object of investigation by such well known scientists as Swedberg 1925, Lizegang, 1926, Pringsheim, 1928, Tizelius, 1930, And Tayshler, 1931 (all spellings of names are a wild guess - phontic in Russian, according to how they think it sounds).

These authors observed violet, light blue and yellowish blue fluorescence colors, its presence in some of the proteins (albumin,

casein, vitellin, elastin, and others) and the absence in the others (albumine of milk, fibrin), its dependence upon the aggregate state (powder or solution), increased luminescnce in proportion to heating (Prinsheim, Herngross, 1928) or radiation with ultraviolet rays (Wells, 1928-1931; Becker and Szendro, 1931; Wigand, 1929). All this cannot but suggest the idea that the majority of researhh men were dealing not with fluorescence, inherent in the proteins proper as such, but rather with the fluorescence of admixtures or chemically changed components of portions of proteins.

For instance, Wells, 1928, using a nephelometer, observed visually light blue fluorescence of the serum globuline with stimulation of the mercury line to 365 mmk. This luminescence cannot be ascribed to the protein proper, already because of the fact that the red limit of absorption of proteins is situated in the shorter wave region of the spectrum. Moreover, similar luminescence has been observed by the author even in the Aminoacids known to be incapable of luminescence, such as glycocol, glutaminic acid, leucine and alanine. Total depolarization of the fluorescence and intensification in proportion to the ultra violet and Roentgen rays radiation likewise excludes the possibility of its belonging in the protein group. Probably, Wells observed nothing else but the universal blue-green luminescence. However, in a number of cases the authors were still dealing with with the long wave "tail" of true ultraviolet fluorescence of proteins; this was the case, for instance, with the shaded blue luminescence of boiled eggs (Van WARGENINGH AND Heesterman, 1932),

as well as in case pf milk luminescence (Genee - spelling?- and Cutler, 1948).

In a number of work, in the '30's, there was confirmed the property of the albumine (protein) bodies to post-luminescence, already mentioned by Nachari (spelling?). In 1933, Hoshijima found that human cartilages, nails and tendons showed marked post-luminescnce after their irradiation with a quartz lamp, whereas other tissues and organs (liver, heart, etc.) did not produce any such post-luminescnce. Two years later (1935), Leighton observed with dark-adapted eye the phosphorescence of the abdominal skin of frog and of human hand which lasted 2-4 sec. In 1937, a vast numberf of articles of biological origin became the object of investigation by Giese and Leighton. These authors found that the lens, stomach, kidney, muscles, blood and skin on the back of frog are incapable of post lumunescnce. The chitin of the sponge gives a short lasting post fluorescence. On the other hand, such articles rich in protein as compact finger nails, birds bills, bones, mollusk shells show quite long lasting post luminescence, dying away in 20-25 sec. These authors made a qualitative evaluation of the spectrum of stimulation of such radiation and they found that it is most actively stimulated by a light of 280 (!) mmk.

Unfortunately, these authors made no attempts to associate the observed radiation with any molecular substratum. In 1940 Reeder and Nelson made a certain step forward in the explanation of the protein property to luminesce; they tested

many proteins: casein, gluteline, gliadin, blood fibrin, gelatine, egg albumine, zein, keratin of hair and fur. Even though the white-blue fluorescence of solid proteins and the blue-green fluorescence of dissolved proteins they observed could not be ascribed to the primary luminescnce of proteins, - inasmuch as the luminescnce could be effectively stimukated by the spectral region of 310-400 mmk, situated outside of the band of protein absorption, and became weak a after the dialysis of specimens, nevertheless these authors were first to observe the causative Association between protein luminescence and their tryptophane content. In this work, they have mentioned, for instance, marked increase in intensity of visual fluorescence of protein during their hydrolysis only in the proteins which contained tryptophane. At the same time, tyrosine and tryptophane were the only aminoacids which gave blue fluorescence after boiling in diluted hydrochloric acid. In this manner, correctly associating the secondary, induced fluorescence with tryptophane, Reeder and Nelson still were far from discovering the primary ultraviolet fluorescence of native proteins.

In 1952, Debye and Edwards, studies a number of proteins and 18 aminoacids at the temperature of liquid nitrogen, and did not find any fluorescence in any of these objects in the visible region of the spectra, although they did notice marked phosphorescence of the aromatic aminoacids, proteins and certain microbic cells. Weber, in 1953, came close to the understanding of

of the possible nature of fluorescence of proteins, when, based on general considerations on the properties of the aromatic aminoacids and the position of their maximum absorption, he assumed that, first of all, protein fluorescence must be situated in the ultraviolet portion of the spectrum, and, in the second place, it should be conditioned precisely by the aromatic aminoacids.

In 1955, Szent-Gyorgyi observed light blue fluorescence of the eye lens, stimulated by short wave ultraviolet light and demonstrated that its carrier is the protein of euglobulin. Although he assumed correctly that the fluorescence capacity is a common property of proteins; and observed it likewise in the epithelium of the cornea and the skin, and mentioned that possibly there may exist considerable fluorescence, extending into the ultraviolet portion of the spectrum and, therefore, escaping observation, Szent-Gyorgyi still proposed a poorly founded conjecture on the protein molecule as a whole being the carrier of luminescence. A retrospective look at his experiments now permits us to understand that Szent-Gyorgyi, apparently, was dealing with long wave portion of the spectra of tryptophane fluorescence of proteins. However, having discovered considerable decrease in the intensity of fluorescence following heat denaturation, he incorrectly associated this fact with the existence of energy levels, common for all native protein molecules which disappears after disruption of the macromolecule configuration ("conformation"). Now it is clear that

that the weakened intensity of fluorescence in Szent-Gyoryi's experiments could have been caused by the high sensitivity of tryptophane as the main center of luminescence to the changes in protein structure. In this manner, by the middle 50's the problem of ultraviolet fluorescence of proteins was literally hanging in the air. Therefore, it is not surprising that the ultraviolet fluorescence of proteins precisely at that time was independently discovered by three groups of authors in the USA and the USSR. Udenfriend (1962), presenting in his well known monograph, the history of the discovery of the ultraviolet fluorescence of proteins, Udenfriend (1962) associates the beginning of studies on ultraviolet fluorescence of proteins with the works of Shore and Pardee (1956) and Konev (1957). However, historically, this is not quite so accurate. Although the author, along with Shore and Pardee, at that time did not know either about each other's works, nor about the works of Duggan and Udenfriend (1956), still the first evaluations of the ultraviolet fluorewcence of proteins rightfully belong to them (are theirs). They were the ones who recorded the fluorescence spectra of human blood serum with its maximum at 360 mmk (without correction for the spectral sensitivity of the equipment). Again they were the ones who presented some arguments in favor of the fact that tryptophane is the center of fluorescence of the serum. Among the arguments, there were mentioned similarity in the fluorescence spectra and the close course of extinction of tryptophane and blood serum hydrolysator by such substances as non organic nitrite, thiosulfate and potassium permanganate.

Soon after this, Shore and Pardee (1956) showed protein fluorescence and that of the aromatic aminoacids, the maximum of which, judging by the light filters used, should be situated somewhere betwee 300-400 mmk; they measured the quantum output of fluorescence, obtaining lowered rates of quantum output: for proteins, 1 - 2 °/o. Among the artefacts, we should also mention the detected drop in the quantum outputs of protein fluorescence and that of aromatic aminoacids, in proportion to decreased wave length of the stimulating light.

In 1956-57, Vladimirov and Konev (Konev, 1956, 1957; Vladimirov and Konev, 1957, 1959_ carried out their first studies of the protein ultraviolet luminescnee/ (aAccording to) From these studies, the fluorescence spectra had marked tryptophane maximum around 350 mmk. The stimulation spectra had the aromatic tryptophane maximum at 280 mmk, and a secondary additional maximum at 240 mmk (Konev, 1957). This maximum was ascribed to the peptid absorption, but subsequent studies sgowed that it represented an artefact. The exclusive aromatic nature of protein fluorescence was definitely proven in the experiements in which no fluorescence was found in proteins, not containing aromatic aminoacids - clupeine, sturnine. The discovered constancy of the protein fluorescence spectra, under stimulation by various length waves showed that, independent of the nature of the centers, which originally absorbed the light quanta, the same centers of tryptophane radiation participate in the fluorescence (Konev, 1957).

From then on, the fixm ultraviolet luminescence of the

protein became the objecy of research studies in many laboratories, both in the Soviet Union (Vladimirov, Barenboim, Sapezhinskii, Konev et al), and abroad (Weber and Teale, Szent-Gyorgyi et al, Cowgill, Steiner Edelhoch and many others).

At the present time, we may take it as having been established that only three aromatic aminoacids which possess luminescence property in free state participate in the formation of luorescence spectra and in the phosphorescence of proteins. These are tryptophane, tyrosine and phenylalanin. Therefore, we shall begin review of luminescence of proteins precisely with the luminescance of these aminoacids in their free state.

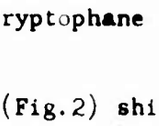
Chapter 1. Electron Stimulated State of Monomers

Tryptophane

Fluorescence Spectra

The tryptophane fluorescence spectrum, in aqueous solution, (Fig. 1) represents a wide non-strucutred (amorphous) band with a maximum at 348 mmk and semiwidth of 60 mmk (Teale and Weber, 1957). The shape of the tryptophane fluorescence spectrum and the position of the maximum are determined mostly by the indol ring of the molecule, without any marked contribution from the lateral groups, since both the indol, and its various derivatives - indolyl-propionic acid, tryptAmine, serotonine, - have practically identicAl spectra of fluorescence.

In favor of little influence on the position of the fluorescence band from the lateral groups is also the fact of a slight shift pf the fluorescence maximum, with changed pH of

the medium (Vladimirov, Li Chin-Go, 1962) R - C - COOH have
a maximum f 347 mmk.
R - C -COO⁻ -353 mmk and R - C -COO⁻-360 mmk. Considerably
greater effect is caused by ionization by the imin group
(in stimulated state). Tryptophane in the ion form  has maximum fluorescence (Fig.2) shifted into the visible region of the spectrum - up to 420 mmk (M K nev, Chernitskii, 1964).

Attention is drawn to two peculiarities of the fluorescence of the aqueous solutions of tryptophane: first, the absence of structure in the fluorescence spectrum, whereas both the absorption spectrum and the fluorescence spectrum, associated with the same systems of fluctuating equations, are definitely structured, and, in the second place, marked Stokes shifting of the fluorescence spectrum - or the order of 70 mmk.

Inas much as, all electron-fluctuating transitions have been solved both for the tryptophane molecule and fpr indol, belonging to the point group of symmetry C_s, it may be assumed that the main reason for the lack of structure in the truptophane band fluorescence in aqueous solutions if its great propensity to interact with the surrounding medium in stimulated state. Therefore, to demonstrate the molecular stucture, one should first of all review the spectra of absorption and fluorescence of tryptOphane in non polar dissolvents (solvents). In view of poor solubility of tryptophane in non polar solvents,

the principal measurements were done with indol.

Fig. 1.

Spetrum of stimulation (1) and fluorescence spectrum (2) of tryptophane in aqueous solution (Teale and Weber, 1957).

One can see in Fig. ⌣ that the absorption spectra of indol in n. hexane have their maximums at 261, 266, 279, 287 mmk and have shoulders (arms) at 271 and 276 mmk. Contrary to Weber (1961), who observed for indol in hexane, a non structured band of fluorescence with maximums at 325 mmk., Chernitskii and Konev (1964) obtained a good structured spectrum of fluorescence with maximums at 288 and 299.5 mmk. and bends (crests?) at 296 and 308 mmk. The absorption spectra and fluorescence spectra, even in such neutral solvent as hexane (Fig. 4), possess no mirror symmetry. At the same time, the aborption and luorescence spectra of a compound kindred to indol and tryptophane - carbazol, which has well separated individual bands ^1L_a and ^1L_b (Fig. 5), are perfectly symmetrical. The absence of such symmetry in indol, in which the bands ^1L_a abd ^1L_b are close to each other, serves as an indicayion that in the formation of the long wave band of the absorption spectrum and fluorescence spectrum both electron passages (transitions) participate at

the same time, but in different ratios. In order to solve the question, which elements in the finer structure should be considered with the variation sublevels of ^1L_a transition (passing-over), and which to the variation sublevels of ^1L_a of the electron level, we may use the following experimental procedure. It has been shown by a number of authors that the formation of hydrogen bond between the molecules of the phenols (Coggeshall, Lang, 1948; Mizushima and others, 1955; Nagakura, baba, 1952), of pyridoxines (Brealey and Kasha, 1955), of naphthols (Nagakura and Gouterman, 1957), and anthrols (SSuzuki and Baba, 1963) and solvent molecules is accompanied by a uniform shoft of the entire long wave band of absorption for these substances. In a number of cases, then, the extent of the shift of the maximum of absorption of molecules corresponds to the e ergy of formation of hydrogen bonds (Brealey amd Kasha, 1955). In a more general way, the amount of the shift of the pure electron transfer (passage) of ΔV_{sym} corresponds to the difference in the energy of formation of hydrogen bond in the stimulated and in the basic states (Pimentel, 1957).

Fig. 2. Indol fluorescence spectra (a) and that of tryptophane (b) at room temperature.
1 - in neutral water solutions. 2 - in mixture of 4°/o NaOH and 0.5°/o formaldehyd; 3 - 9n 4°/o NaOH solution

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Chernitskii and Konev, 1964, observed similar ratios also for the indol related compound, the carbazole. Both vibrating (oscillating) maximumd of the electron transit of the long wave band of carbazole absorption

of carbazole absorption in the transition from the hexane solutions to the alcohol in which the hydrogen of the nitrogen atom of the imminogroup enters into a hydrogen bond, are shifted into the long wave side for an even number of reverse (opposite) centimeters. Similarly, both maximums of the fluorescence spectrum (Table 1) are shjfted for the same number of opposite centimeters.

Fig. 3. Indol absorption spectra in water (7) and in mixtures of hexane with n. buthanoil (1-6). N. buyhanol concentrations (in volumetrical percentages) are respectively:
1 - 0; 2 - 1; 3 - 3; 4 - 5; 5 - 20; 6 - 100

Possessing an iminogroup, indol, just like carbazole, is capable of formin hydrogen bonds with the solvents. Dodd and Stephenson, 1959, with the method of infrared spectroscopy observed the shift in NH vibrations, corresponding to the formation of hydrogen bobd, while Sannigrahi and Chandra, 1963, determined the constant of equilibrium of the complexes with hydrogen bonds, by the increase of obsorption on the long wave drop of absorption band of 2-methyl-indol with addition of cyclohexane ether solutions and di-oxane.

With gradual addition of n. buthyl alcohol to the hexane solution os indol, Chernitskii and Konev, 1964, as als in the case of carbazole, observed gradual yransformation of the spectra. The isobest (? word not in dictionary) point at 268 mmk (Fig. 3) indicates the presence of two forms of indol molecules in the binary alcohol-acetone solution - the neutral one and one bound

with alcohol in hydrogen bond. As also in the case of carbazole, all maximums of absorption are shifted into the long wave side, and this indicates the unquestionable belonging of the entire band of absorption ti the system pi - pi of the transition.

Table 1

Position of maximums of absorption and fluorescence of carbazole in hexane and in butyl alcohol.

Absorption, cm^{-1}				Fluorescence, cm^{-1}			
ν hexane	ν alcohol	$\Delta\nu$	$\Delta\nu$cp	ν hexane	ν alcohol	$\Delta\nu$	$\Delta\nu$ cp
30180	29600	580	580	30030	29100	930	920
31430	30840	590		28740	27830	920	

cp if Russian
sr if Eng.

However, the more important to us feature (or peculiarity) of the spectral shifts, which accompanies the transfer from the hexane to the alcohol solution, consists in the fact that the elements in the structure of the long wave band of absorption are displaced by different absolute degree. The short wave maximums undergo a more marked red shift - 700 cm^{-1}, than the long wave, at 287 and 279 mmk which are only displaced by 150 opposite centimeters. From the non identical maximum of absorption shifts, with the formation of hydrogen bond, we derive an important conclusion: the long wave band of absorption of indol (and hence, that of tryptophane) is formed by two electron shirts (transfers). One of this is $^1L_b \rightarrow A$ with variable maximums at 287 and 279 mmk, at a distance of 1000 cm^{-1} one from the others; the other one, which is shorter wave is ^1L_a and forms a series of electron

bands, starting from 276 cm^{-1} and situated at shorter distance one from the other - 700 cm^{-1}. As we shall see, this conclusion is well supported by polarizational measurements. The various effect of the hydrogen bond upon the position of the maximums, corresponding to the electron transfers $^1L_a \leftarrow A$ and $^1L_b \leftarrow A$, permits us to conclude as to the orientation of these moments with reference to the "skeleton" of the molecule. This possibility is based on the following literary data. In the derivatives of anthracin and naphthalene, introduction of lateral substitutes into the molecule produces greater effect on those electron transfers, the direction of the moment of which passes through this substitute (Jpnes, 1947; Baba and Suzuki, 1962). In a similar manner, the spectra are Affected not only by the substitutes but likewise by the hydrogen bonds (Suzuki and Baba, 1963).

The same regular patterns are observed also in a compound, kindred to tryptophane and indol, the cabazole. The substitution of hydrogen by in the immuno group by various radicals causes a marked shift in the band ^1L_a, the oscillator of which is oriented along the shorter axis of the molecule, i.e. passes through the nitrogen atom (Platt, 1951; Schuett, Zimmermann, 1963). And, conversely, the substitutes have a weak effect on the ^1L_b transfer, associated with the long Axis of the molecule (Omel'chenko, Pukh-kareva, Bogomolov, 1957). The same is true for the effect of hydrogen bond on the absorption bands, conditioned by the transfers ^1La and ^1L_b: with t e formation of hydrogen bonds between the OH-groups of alcohol and NH of carbazole, the ^1L_a is shifted to 580 cm^{-1}, whereas ^1L_b, the moment of the transfer of which does

not pass through the immunogroup, is onyl shifted by 370 cm^{-1} (Chernitskii, Komev, 1964). These ratios may be useful in the evaluation of orientation of scillators of indol and tryptophane absorption: the greater spectral shift in the short wave maximums indicates (^1L_a) orientation of their corresp=onding oscillators along the shorter axis of the molecule. The little shifted (displaced) long wave maximums of indol 279 and 287 mmk correspond to the electron transfer $^1L_b \leftarrow A$, oriented along the long axis of the molecule.

Fig. 4.

Absorption spectra (1) and fluorescence spectra (2) of indol in n. hexane ($t=20^oC$, $c=5\cdot10^{-6}M/l$)

Fig. 5.

Absorption and fluorescence spectra of carbazole in n. butanole (a) and in n. hexane (b).

Now, let us consider hhe effect of polar additions on the indol fluorescence spectra. A greater shift into the long wave side of the vibration maximums of the transfer ^1L_a, as compared to the vibrztion maximums of ^1L_b gives additional proof of the complex nature of the indol fluorescence spectrum, in the formation of which participate not one, as in the majority of the remani remaining moleculres of organic substances, but two electron trAnsfers. With addition of indol/to the hexane solution, the of n. butyl alcohol maximum at 268 mmk is greatly diminished in intensity, at the same time only insignificantly shifting into the long wave side

(Fig. 6). The situation is different in case of maximums at 299.5 and 308 mmk, which are shifted much more markedly into the long wave side by 8 - 8.5 mmk. In the indolyl-propione acid (deamidized tryptophane) this shift is still more marked - 14 mmk. Returning to theproblem of the symmetry in the absorption and flucrescence spectra of indol in hexane solution, and taking into consideration the above proposed feature that their elements belong to different electron transfers, it may be assumed that the absence of symmetry in these spectra reflects different relative participation of the two electron transfers in the formation of fluorescence spectrum And a different amount of Stokes shift (displacement) for each one of them. Nevertheless, in a number if elements, in the structure of absorption spectrum and fluorescence spectrum, there is observed fine frequency conformity. Almost resonAnce-like coincidence of fluorescence band at ~~282xmmkxwithxthexmaxtmumxafxabsarptian~~ at 288 mmk with maximum of absorption at 287 mmk should be ascribed to ^1L_b transfer. These bands include in themselves 0-0 tr^nsger (pasaage) of the band ^1L_b. The same electron transfer forms maximum at 279 mmk (33800 cm^{-1}) in absorption and in the shoulder (arm) of 296 mmk (37800)in the fluorescence. According to the assumption, the vibration sublevel 33800 cm^{-1} in absorption and sublevel 35800 cm^{-1} in fluorescence are at equal energy distances from their 0-0 transfer at 287.5 mmk (34800 cm^{-1}): both in absorption and in fluorescence at by 1000 cm^{-1}. In this manner, the fluof fluorescence band of ^1L_b has an insignificant Stokes dislacement (shift). The fluorescence band ^1L_a had a greater shift.

According to their frequencies, it is possible to compare the main maximum of fluorescence at 299.5 mmkk(33400 cm^{-1}) and the bend at 308 mmk (32600 cm^{-1}) maximums in absorption at 276 mmk (36200 cm^{-1}) and 271 mmk (36800 cm^{-1}). These elements are separated, both in the absorption spectrum and in the fluorescence spectrum by an identical frequency interval of 800 cm^{-1}. Based on the law of the symmetry of the absorption and fluorescence spectrum, the 0 0 transfer of the band ^1L_a is situated with 34800 cm^{-1} i.e. it almost coincides with the pure electron transfer of the band ^1L_b (34820 cm^{-1}). It is possible to obtain better structured quasi-linear spectra for indol in cyclohexane at 77°K. In this case, the semi-width of the most intensive lines does not exceed 120 cm^{-1} (Kembrovskii, Bobrovich, Konev, 1965). Just as well structurally built is the stimulation spectrum (absorption) which coincides well with the absorption spectrum of the indol vapors (H les - spelling?-, 1963). We present some psectra from our works (Fig. 7). In this illustration, one can see the division in the fluorescence spectrum of the vibration frequencies into two series, one of which has the vibration frequencies of 610, 760, and 1050 cm^{-1}, coinciding with the absorption requencies and (Cohlrausch, 1938), corresponds to $^1L_b \rightarrow A$ transfer with 0-0 frequency at 347.3 cm^{-1}; the second series with the same vibration frequencies is manifested with the same vibration frequencies in calculating the vibration quanta from the band 33390 cm^{-1}, as 0-0 of transfer of ^1L_a in emission, the 0-0 band of $^1L_a \leftarrow A$ of transfer in absorption is not apparent because of the effect of the Frank-Condon principle.

Fig. 6. Indol fluorescence spectra in water (9) and in mixtures of n. hexane with n. butanol (3,5,6,7) at t=20°C, as well in n. butanol at t=-196°C. For the curves 1,3,4,6, and 7, concentration of n. butanol (in volumetrical percentages); 1 -0; 3-0.7; 5-2.5;6-5;7-100

Fig. 7. Indol in cyclohexane t= -196°C: 1 - spectra of fluorescence stimulation; 2 - fluorescence; 3- phosphorescence.

Doubling of the vibration frequencies in the fluorescence spectrum indicates the complex nature of the fluorescence spectrum, consisting of ^1L_a and ^1L_b frequencoes. A though for tryptophane itself it is not possible to get structured spectra, nevertheless the tryptophane residues, included into the composition of the amylase protein crystals at the temperature of liquid nitrogen, disclose in the fluorescence spectra both components ^1L_a and ^1L_b.

The Above statements show that thed fluorescence spectrum of a real indol and tryptophane moleculre, placed into a strongly polar (aqueous) environment, has lityle resemblance to the fluorescence spectrum of an "isolated" moleculre, in the conditions in which the outside environment causes minimum possible stimulation of energy levels of the molecule. Then what is the elementary nature of the intereffects of the tryptophane molecule with the environment which result in such strong distortion of the molecule energy? First of all, the organic molecules, incapAble of forming water bonds, show, as a rule, identical position of fluorescence maximums in the hexane solutions and in n.

butyl alcohol at low temperature. On the contrary, in the substances capable of forming hydrogen bonds, as for instance in naphthalene and anthracene derivatives (Suzuki and Baba, 1963) or in carbazole (Chernitskii, Konev, 1964), their formation is accompanied by a shift in the fluorescence spectrum into the long wave side, as a whole, with the preservation of the shape (form). In indol and tryptophane, the long wave shift is accompanied with a supplementary effect - that of the disappearance of short wave maximums of fluorescence. Hence, it follows that the first cause for the shift of the fluorescence spectra into the long wave side in any polar medium is the establishment of hydrogen bond between the iminogroup and the colecules of the solvent. But, inasmuch as the hydrogen bond is formed as though with two different electron-stimulated molecules and displaces their spectra to various distances, then as a result of this, the 0-0 passage (transfer, span) of ^1L_a appears in the longer wave zone than the passage ^1L_b ; the energy distance between them is so much increased that the indol and tryptophane molecules considerably lose their "peculiarity: - they begin to give light, just like other substances, but only from one the lowest electron level. In this manner, along with the long wave shift of fluorescence, the hydrogen bond weakens the intensity of the fluorescence of ^1L_b.

don't know the exact term in electronics

conversion

However, the formation of hydrogen bond does not deplete possible intereffect of tryptophane and indol molecules and the solvent. Already with the addition of small amounts of butyl alcohol to n hexane solutions (tenth portions of the volumetrical

percentage) which cannot alter macroscopically the dielectrical properties of the medium, there is observed a progressive displacement (shift, phase difference) of the fluorescence spectra into the long wave side. The shift of fluorescence spectra with the change from the hexane solutions to the alcohol solutions is, for indol, from 289.5 to 340 mmkk and for the ~~indol~~ indolyl-propionic acid from 305 to 345 mmk.

In this shift, along with the formation of hydrogen bond, there also participate some relaxing manifestations of the solvent (Cherkasov, 1960, 1962). In their basic state, the power of the intermolecular effect causes certain prientation of the polar molecules (of alcohol) around tryptphane or indol. With change of the molecule into single stimulated condition, the extent and the direction of the dipolar moment are changed. Changes in the physical properties of the molecule are accompanied by changes in the force and in geometry of the intereffect with the neighbor molecures of the solvent and by corresponding changes in the equilibrium orientation. Among these, when the time of relaxation is commeasurate with the period of life of stimulated state (of the order ot 10^{-8} - 10^{-9} sec.), there appears a whole set of stimulated molecules, found at the moment of radiation in at various stages of relaxation or, in quite general form, in different states of ~~xxxxxxxx~~ of intereffect with the near-by molecules of the solvent. At the same time, the relaxation processes of the solvent, along with the widening of the band of radiation, must be responsible for the fact that the long wave zone of the spectrum will be formed by luminescence of molecules with the

single?
"singletnyi"
not Russian word

longest life span which which there will take place relaxation phenomena. As a matter of fact, addition of extinguishers to the hexane solutions of indol and indolyl-propionin acid is not accompanied by changes in the form of the fluorescence spectra, whereas the same solutions containing a small admixture of butyl alcohol, undergo a certain short wave shift of the fluorescence spectrum. All this indicates that in the solvents which contain polar molecules, various centers participate in fluorescence, and the long wave portion of the spectrum is conditioned by luminescence of the molecules with the greatest life span. The relaxation nature of the long wave displacement, widening and (loosening) loss of structure diffusion of the structure of the fluorescence spectrum in the presence of small quantities of polar molecules is manifested in that after elimination of possible relaxation by means of increasing of viscosity or lowering of temperature (cooling of the alcohol solution down to their freezing point and lower), there are observed a short wave shift of the spectrum and the Appearance of vibratory structure. In a similar manner, increased viscosity of the glycerine solvations with lowering of temperature results in a certain short wave shift of the fluorescence spectra.

Thus, the specific intereffect (using the terminology of Bakhshiev and Neporent, 1960) which occurs between the molecule of the dissolved substance and one or several of surrounding particles of the medium, are apparent in case of indol and its derivatives in the formation of hydrogen bonds and orientation solvency with relaxation of solvate membrane with reference to the stimulated molecule. These microintereffects are quite power-

ful and results in three consequences: 1) long wave shift of the fluorescence spectra, 2) their widening, and 3) loss of structure.

It is quite understandable that, along with the specific (individual) interfeect, of the indol and tryptophane molecules as well as the other substances, are capable of participating in the universal intereffects, associated with the influence upon the dissolved molecule of the entire complex combination of the surrounding molecules. Essentially, because of the influence of the dielectrocal properties of the solvents, the maximums of fluorescence, in transition from the alcohol solution to aqueous change solutions, are shifted from 330 to 342 mmk. in indol, and from 345 to 360 mmk. in indolyl-propionic acid and from 338 to 350 mmk in tryptophane (Fig. 8). The universal effect of the solvent, its dielectrical properties, upon the position of the tryptophane maximum fluorescnce was shown by Teale (1960), in an example of water-dioxane solutions of glycyl of tryptophane. The position of the maximum was displaced into the short wAve side, corresponding to the decreased dielectrical constAnt of the binary solvent.

Fig. 8. Tryptophane fluorescence spectra in water (9), in dioxane (8) and in n. butyl butanol at t=20°C (7), as well as in n. butanol at t= - 196°C (10).

In the pure dioxane solution, tryptophane has its maximum at 329 mmk instead of 350 mmk in the Aqueous solution. Apparently, the position of the maximum fluorescence and the form (shape) of the spectrum, under real conditions, is determined by the influence (effect) of all three main types of intereffect:

universal (influence of the integral field of the solvent on the dipolar moments of tryptophane molecules in stimulated condition (state)) and two specific ones - effect of the hydrogen bond and that of the relaxation of the solvent. The greater influence of the intereffect with the environment on the position of the levels of stimulated state (greater shifts in the fluorescence spectra than in tje absorption spectra) than that of the basic one is determined by the circumstance that the dipolar moment of the stimulated molecule increases markedly and, as a result, the power of intereffect with the environment also increases (intereffect of constant or induced charges).

In conclusion, let us review the frequently discussed problem of tryptophane fluorescence in the visible zone of the spectrum. Steel and Szent-Gyoryi, 1958, Isenberg and Szent-Gyorgyi, 1958, Fujimori,,1960, found for the concentrated typ tryptophane solutions ($5 \cdot 10^{-3}$ - 10^{-2} M/l) in glucose at low temperatures. along with the usual fluorescence (325 mmk and phosphorescence with three maximums in the region of 400-500 mmk) also an unusual fluorescence at 450 mmk. with its corresponding phosphorescence at 500 mmk, which could be stimulated by low effective light with lambda equal to 365 mmk. If we do not consider that this luminescnce is conditioned by admisxtures, then, we could perhaps think they belong to the n ---- pi passages in tryptophane molecules which have a strong reciprocal intereffect (and possibly some additions) through the hydrogen iminogroups. This is the more probable, since, according to the observations by Steel and Szent-Gyorgi,

1958, in this zone it is possible to demonstrate on the concentrated solutions some hints to extremely weak maximum of absorption, on the one hand, and that Fujimori, 1960, noted disappearance of luminescence 450 and 500 mmk in 8 M hydrochloric acid, on the other hand. Phosphorescence with a maximum at 500 mmk was later observed by us with Chernitskii in sugar candy tryptophane at room temperature. Lehrer and Fasman, 1964, reported on exaimeric (?) fluorescence of topytryptophane at 470 mmk.

Quantum Output of Fluorescence and Its Dependence Upon the Structure of Tryptophane Molecule and Upon Outside Conditions

According to Teale and Weber, 1957, the quantum output of fluorescence of neutral aqueous solutions of tryptophane is 0.2 for the entire region of stimulation 210 - 300 mmk., i.e. the fluorescence stimulation spectra mmk coincide with the absorption spectrum (Fig. 1). The quantum output of fluorescence is sensitive to the ionization state of functional groupings. White, 1959, studied the dependence of the qunatum output of tryptophane fluorescence upon ghtg the pH of the medium and found a very marked ratio, represented in Fig. 9, which reflects the processes of ionization in this aminoacid. Similar curves of titration of tryptophane fluorescence have been obtained later by many ot er authors - Weber, 1961; Vladimirov and Li-Chin-go, 1962; Cowgill, 1963 A; Konev and Chernitskii, 1964.

The non ionized carboxyl group COOOH is intensely extinguishing in the tryptophane molecule, then the aminogroup assimilating protone, and the iminogroup, which lost protone. The most effective extinguishers of fluorescence are the first and the third

groupings. As we know, tryptophane, like any aminoacid, represents an ampholyte, capable of dissociating both as an acid and as a base

$$H_3N\text{-}R\text{-}COO^- + H^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} H + N\text{-}R\text{-}COOH$$

$$+ \; {}_3HN\text{-}R\text{-}COO^- \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} {}_2HN\text{-}R\text{-}COO^- + H^+$$

$$K_1 = \frac{/{}^+{}_3HN\text{-}R\text{-}COO^-//H^+/}{/{}^+{}_3HN\text{-}R\text{-}COOH/}; \qquad K_2 = \frac{/{}_2HN\text{-}R\text{-}COO^-//H^+/}{/{}^+{}_3HN\text{-}R\text{-}COO^-/}$$

pK_1, or the value f pH, at which the concentrations $/{}^+{}_3HN\text{-}R\text{-}COO^-/$ and $/{}^+{}_3HN\text{-}R\text{-}COOH/$ are equal, i.e. the pH value (or rate), with which one half of the molecules have a charged, and the other half a carboxyl group; this is for tryptophane 2.3:pK_2 of aminogoup situated in the alkaline medium at 9.38. Therefore, it is reasily understood that changes in the quantum outputs, conditioned by ionization of lateral groupings of tryptophane, must occur near these two values of pK. Actually, in proportion oo the increased pH rate, beginning with the most acid solutions, there is observed a two-step growth in the quantum output, reflecting the course of dissociation of lateral groups. In the Acid medium, there is observed the most effective extinction of luminescence, since here the lateral groups are in the condition of COOH and $-NH^+_3$. Increase in the quantum output in proportion to the increase in pH from pF=2.3 (the point corresponding to the middle of the curve of increase of output) completely c orresponds to the found amperometrical pK of dissociation of the carboxyl group - 2.3.

FIG. 9. Dependence (or ratio) of the quantum output of fluorescence of tryptophane (1), tyrosine (2) and phenylalanine (3) upon the pH of the medium. Rates for phenylalanine are increased 10 times (1 and 2 - White; 195°; 3 - Feitelson, 1964).

According to the data of Cowgill, 1963, tryptophane with non ionized COOH group and protonized aminogroup has a low quantum output of fluorescence, 0.085 (Table 2).

The mechanism of acid extinction of tryptophane fluorescence is unequivocally demonstrated not alone by t e coincidence of the curves of titration of carboxyl group and "fluorescence", but also by the absence of extinction - shift of pK fluorescence to 0.5 - in compounds withoot carboxyl grouping (indol) or in compounds in which this grouping is in chemically bound state - methyl ether N-acethyl-tryptophane. In the interval of pH 4 - 8, all tryptophane molecules in the solution are in the condition of $^{+}{}_{3}HN\text{-}R\text{-}COO^{-}$ which corresponds to constant quantum output 0.2, independent from pH (Teale and Weber, 1957; White, 1959; Cowgill, 1963). In a more alkaline region, there begins transition (transfer) of the protonized aminogroup into the neutral $^{+}{}_{3}HN\text{-}R\text{-}COO^{-} \rightarrow {}_{2}HN\text{-}ROCOO^{-}$, accompaznied by further growth of the quantum output The pF of this transfer (passage) corresponds to pK of dissociation of aminogroup, although with a certain shift into the alkaline side: pF=9.4 (White, 1959; 9.5 (Cowgill, 1963); 9.4 (Vladimirov, Li Chin-Go, 1962), whereas pK is equal to 9.38 (French, Edsall, 1954) Tryptophane in the ion form ${}_{2}HN\text{-}R\text{-}COO^{-}$ has quantum output 0.51 (Cowgill, 1963). This quantum output is maximum for aqueous solutions and is observed with pH 10.9.

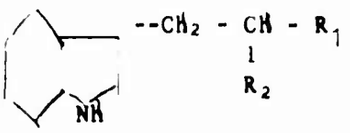
The fact that the second step in the curve of increase of quantum output in the area of pH 8-11 is conditioned by the dissociation of the aminogroup may be proven also by the constancy of the quantum output in the compounds, not having any aminogroup of N-methyl-indol (White, 1959), indol and indolyl-propionic acid (Fig. 10) (Konev, Chernitskii, 1964) or in the compounds in which hydrogen of the aminogroup is substituted by another radical - the methyl ether N-acethyl-tryptophane (cowgill, 1963). The maximum possible rates of the quantum output 0.51 are preserved in tryptophane in a wide diapasone of pH 4 -12 in the case, if the aminogroup is blocked with the addition of formaldehyd in an Alkaline medium (Fig. 11), where chemical bonds of the type R - n = CH_2 or R - NH - CH_2OH (Konev, Cernitskii, 1964) arise. Then formaldehyd has no effect on the quantum output of indol and indolyl-propionic acid which do not contain aminogroup (with the exception of the area of pH 11-13, where effects of blocking of the iminogrpup are already felt).

Fig. 10. Dependence of quantum output of fluorescenae: indol (a) and indolyl-propionic acid (b) upon the pH of the medium in aqueous solution (1), immediately following 1°/o formaldehyd (2), in 1°/o formaldehyd 1 hour after it has been added (3).

Fig. 11. Dependence of quantum output of fluorescence of tryptophane upon the pH in the medium:
1 - in aqueous solution; 2 - in 1°/o formaldehyd immediately after its is added; 3 - in 2°/o formaldehyd immediately as added; 4 - in 1°/o formaldehyd one day after addition.

Table 2

The Effect of Lateral Substitutes on the Quantum Output of Fluorescence in Tryptophane Derivatives



Compound	R_1	R_2	B
Tryptophane	$-COO^-$	$-NH_2$	0.51
N-acethyl-tryptophane	$-COO^-$	$-NH-CO-CH_3$	0.23
Amide tryptophane	$-CO-NH_2$	$-NH_2$	0.28
Tryptophane	$-COO^-$	$-\overset{+}{N}H_3$	0.20
N-acethyl-tryptophane	$-COO^-$	$-NH-CO-CH_3$	0.128
Tryptophane	$-COOH$	$-\overset{+}{N}H_3$	0.085
Methyl ether acetyl-tryptophane	$-COOCH_3$	$-NH-CO-CH_3$	0.080
Ethyl Ether Trypto-phane	$-COOC_2H_5$	$-NH_2$	0.076
Ethyl ether trypto-phane	$-COOC_2H_5$	$-\overset{+}{N}H_3$	0.032
Glycyl-tryptophane	$-COO^-$	$-NH-CO-CH_2-NH_3$	0.095
Glycyl-tryptophane	$-COO^-$	$-NH-CO-CH_2-\overset{+}{N}H_3$	0.057
Glycyl-tryptophane	$-COOH$	$-NH-CO-CH_2-\overset{+}{N}H_3$	0.022

In literature there are two points of view, quite close, as the the mechanism of changes in the quantum output with ionization of carboxyl and aminogroups of tryptophane. According to White, 1959, whose opinion is also shared by Weber, 1961, extinction with the groups COOH and Nh_2 has a common mechanism - extinction by *tpt* pron which belongs in these groupings; it is attracted to the indol ring in stimulated state. Cowgill, 1963, bases his views on the electron negativity of the lateral groupings, i.e. their capacity to attract the electrons of the indol,ring. In proportion to increase in the number of neutral methyl groupings,

between the indol ring and the electron negative substitute, the extent of its effect upon the quantum output is gradually diminished: the quantum outputs of indol $-COO^-$, indol $-Ch_2-COO^-$ and indol $-Ch_2-Ch(Nh_2)-COO^-$ are 0.24; 0.38 and 0.51 respectively.

The role of the third functional grouping of tryptophane - the iminogroup - is manifested only in strongly alkaline medium, starting with pH 11. Precisely with the presence of this functional grouping is associated extinction of fluorescence in strongly alkaline medium. The role of the mobile hydrogen in the iminogroup is confirmed by the absence of extinction in the strongly alkaline medium in N-methyl-indol (White, 1959), methyl ether N-acethyl-tryptophane (Cowgill, 1963). Decrease in intensity of indol and tryptophane fluorescence is also absent in the case, when the iminogroup is blocked by formaldehyd (Fig. 12, Konev, Chernitskii, 1964).

White has offered the following mechanism for the explanation of alkaline extinction:

$$RNH+h\nu \rightarrow RNH^*$$

$$RNH^*+OH^- \rightarrow RN^-+OH_2$$

$$RN^- \rightarrow RN^- \text{ (non-emissive transfer)}$$

$$RN^-+OH_2 \rightarrow RNH+OH^-$$

After Boaz and Rollefson, 1950, who demonstrated the non emissive character of the transfer (passage) $R^*N^- \rightarrow RN^-$ in alpha-naphthylamide, White considered the extinction with the hydroxyl ions as transfer of H-ion from the nitrogen atom in the stimulated molecule of tryptophane to the ion of OH-solvent. White's point of view is also shared by Weber, 1961, Vladimirov, Li Chin-go,

1962, Udenfriend, 1962. However, this scheme is need of certain elaboration of details.

The first condition in the scheme of White - non dissociation of hthe iminogroup in the principal non stimulated state is beyond doubt. The absence of ionizing form of indol ring in the non stimulated state is confirmed by the coincidence of the curves of alkaline titration of indol and water which indicates the absence of the reaction

$$RNH + OH^- \quad RN^- + H_2O,$$

which would have used additional quantities of alkaline (Konev, Chernistkii, 1964).

Fig. 12. Polarization spectra of indol fluorescence (1), that of N-methyl-indol (2) and tryptophane (3) (Weber, 1960).

Things are different in the second condition of the White scheme - the inability of the ionized ~~gxmm~~ form of the indol ring to fluoresce. Our measurements together with Chernitskii (Konev, Chernitskii, 1964) showed that in 4 ^{o}/o solution of NaOH indol and tryptophane have a fluorescence with quantum output approximately on and a half times lower than in the neutral solutions, and with the maximums situated at 405 and 420 mmk respectively (Fig.2). In this manner, ionization of the iminogroup in stimulated state results not in a simple extinction of luminescence, but to the appearance of some molecules, capable to be luminescent in the longer wave region of the spectrum. Moreover, the quantum output of fluorescence of these molecules has low rate, not because of the slight thermal dissociation of the energy

of electronic stimulation inside of the indol ring, as assumed by White, but as a consequence of kinetic extinction of luminescence of ionized molecule during collisions with the hydroxyl ions. At the temperature of 77°K the fluorescence of alkaline indol, remaining considerably shifted into the long wave side, as compared to the neutral solution (370 mmk instead of 320 mmk), still acquires the initial rates of the quantum output. Therefore, with elimination of kinetic impacts, photodissociation of the iminogroup takes place (the spectrum is shifted), although extinction ceases. This permits us to divide in time the actions of photoionization and extinction, assuming that the tryptophane/molecules, and indol ionized for the iminogroup, are capable of luminescence by themselves, but with excess of alkali become extinguished, colliding with hydroxyl ions. In this manner, and a finer further elaborated form the diagram of fluorescence extinction in strongly alkaline medium, conditioned by the photoionization of the iminogroup of indol and tryptophane with subsequent extinction of luminescnce at the time of collision with hydroxyl ions, will appear as follows (Konev, Chernitskii, 1964):

$$RNH + h\nu_1 \rightarrow RNH$$

$$RNH + OH^- \rightarrow RN + H_2O$$

$$RN^- \rightarrow RN^- + h\nu_2 \text{ (fluorescence)}$$

$$RN^- + OH^- \rightarrow RN^- + OH^- \text{ (extinction)}$$

$$RN^- + H_2O \rightarrow RNH + OH^- .$$

Tryptophane fluorescenee is extinguished with heating (Gally and Edelman, 1962), addition of oxygen (Barenboym, 1963; Barenboym, Domanskii, 1963), of potassium iodide (Vladimirov and Li Chin-go,

1962) and litium bromide (Konev, Bobrovich, 1964). In the last mentioned two cases, extinction is conditioned by easing up of the conversion into the triplet state. The same cause may, apparently explain the weak fluorescence of indol in hexane solutions at low temperatures, when an intense fluorescence is observed

Polarization spectra of tryptophane fluorescence

Polarization spectra of fluorescence with absorption

The polarization spectra of fluorescence for tryptophane And indol absorption have been obtained by Weber in 1960. The spectra were taken in propylene glycol at the temperature of -70 C, fluorescence was stimulated with non polarized light Therefore, in order to get the commonly accepted rates of the degree of polarization P, Weber's figures must be multiplied by the coefficient equal to $\frac{2P_n}{P_n + 1}$ (P_n - degree of polarization during ~~xxxxxxxxxxx~~ ~~xxxhxxxxxxxxxxxxxx~~ stimulation with non polarized light). According to Weber's data, indol, N-methylindol and tryptophane have close polarization spectra with two minimums at 232 ±2 and 290 mmk and two maximums at 265 - 270 and 300 - 305 mmk (Fig. 12).

Low absolute rate of the degree of polarization throughout the entire long wave band of absorption, with the exception only of the long wave margin itself, not specific for molecules with low symmetry are well approximated, as we know, with linear oscillator, and what is most important, discrepancies between hthe polarization spectrum and the absorption spectrum, led Weber to assume the complex nature of the long wave band of absorption, consisting of two oriented at an right angle moments of electron passages (transfers). At the same time, Weber mentions that only one of the two

electron levels participates in radiation; while the second electron level only transmits to it the absorbed energy. Such a system of oscillators of extinction gives a clear idea on the pain details observed in experiments on onthe peculiarities of the polarization spectrum: the higher rates of the degree of polarization - 35^{0}/o to 50^{0}/o in the area (zone) of 300-310 mmk. correspond to light absorption by the oscillator $^{1}L_a$ alone; the minimum at 290 mmk corresponds to maximum absorption by the "negative" oscillator $^{1}L_b$ which lowers the positive osciklator polarization of oscillator $^{1}L_a$; maximum at 265-270 mmk corresponds to the od absorption $^{1}L_a$, as result of which the relative contribution to the absorption by t e oscillator $^{1}L_b$ at this point of the spectrum is diminished and there is pbserved relatively great positive polarization, etc. However, Weber's works do not contain any proof that luminescence is conditioned only by one kind of luminescent molecules. It is conceivable that the pre# sence of molecules from admixture, or another form of tryptophane molecules proper with an absorption band at 290 mmk could cAUSE the appearance in this zone of minimum in the polarization spectrumm1/ of fluorescence. Therefore, the original rate brings the proof that both absorption oscillators belong to the one and the same luminescent molecule.

Chernitskii, Konev and Bobrovich (1963) showed that in the solid solution of p-iyvynii polyvinyl alcohol in the region of 250 - 300 mmk there is observed independence of the tryptophane fluorescence spectra from the length of the wave of the stimulating light and at the same time there is perfectly preserved usual

form of polarization spectrum with a gap in the region of 289-290 mmk (Fig. 13).

Fig. 13. Spectra - dl - of trytophane in the film of polyvinyl alcohol at 20°C:

1 - absorption; 2 - fluofluorescence; 3 - polarization with absorption 9 registration 330 mmk); 4 - the same (registration 295 mmk); 5 - polarized fluorescence after radiation (stimulation 265 mmk) and t= -196°C' 7 -phosphorescence; 8 - polarized phosphorescence after [illegible] absorption (registration 460 mmk); 9 - polarization phosphorescence after radiation (stimulation 265 mmk). 10 - spontaneous polarization phosphorescence after radiation (emission)(stimulation 365 mmk); 11 - spontaneous polarization phosphorescence after absorption (registration 460 mmk).

It was found that the typical form of polarization spectrum appears in almost unchanged for for viscous solvents of different chemical nature: glycerine (Konev, Katibnikov, Lyskova, 1964), sugar rockcandy (Chernitskii, 1964), polyvinyl alcohol (Sevchenko, Konev, Katibnikov, 1963; Chernitskii, K nev, B brovich, 1963)$ These authors obtained similar spectra both for tryptophane and indol.

A second proof that both oscullators of absorption belong to the same molecules was found in the experiments with irradiation of polarization spectra of tryptophane in proportion to increased concentration of it in polyvinyl alcohol. These spectra shpwed that at the point of 289 mmk there takes place the same concentration depolarization, as in the remaining portion of polarization spectrum after absorption within the limits of 240-300 mmk (Fig.14) (K nevm Katibnikov, Lyskova, 1962, 1964; Chernitskii, Konev, Bobrovich, 1963). The concordance of concentration depolarization at various points of the spectrum indicates that at all points f stimulation we

stimulationwe are dealing with identixal molecules in equal absolute concentration.

Similar maniestations are also observed in case of concentration depolarization of phosphorescence. The existence of the oscillator ^1L_b in the long wave band of absorption, particularly graphically may be proven in the experiments with oriented tryptphane (Sevchenko, Konev, Katibnikov, 1963; Chernitskii, Konev, Bobrovich, 1963). Orientation of the molecules may be obtained with mechanical stretching of the membranes (films) of polyvinyl alcohol, activate with tryptophane. Let us consider the following diagram of the experiment: the surface plane of the film (membrane) is at an agle of 45° to the direction of the distribution of the stimulating ray in such a manner that the oscillator ^1L_a is placed vertically, fluorescence is stimulated with the horizontal component of the linearly polarized light, polarization of the fluorescence is measured at its principal maximum at an agle of 90^0 to the same direction of the distribution (spread) of the stimulating light (Fig. 15). With such a diagram it is possible to get a straight answer as to whether the oscillator ^1L_b exists and what sort of absorption spectrum it may form.

As a matter of fact, with such a diagram of experiment, the non oriented portion of the molecule, independently from their oscillatory nature must show the totally depolarized fluorescence throughout the entire absorption spectrum, as this is represented in Table 7 (Fig. 16). Now let us see what sort of polarization will be given by the oriented portion of the tryptophane molecules? If the lon wave band of absorption is conditioned only by one

oscillator ^1L_a, oriented according to the conditions of the experiment vertically, then the oriented tryptophane molecules should not at all be stimulated horizontally by the polarized light and the fluorescence of the film (membrane) should remain totally depolarized as before. The state of affairs will be quite different, if a second oscillator participates in the formation of absorption band, being oriented at a right angle to the first oscillator. This oscillator is situated at an angle of 45° to the vector of stimulating light and, therefore, it takes part in its absorption. Now there will be superimposed on the depolarized, as before, chaotically scattered tryptophane molecules polarized fluorescence, conditioned by the oscillator ^1L_a, in/oriented portion of the molecules.

Fig. 14. Concentration Extinction and Concentration Depolarization of tryptophane fluorescence in solid film of polyvinyl alcohol.

a - dependence of relative quantum output of fluorescence of tryptophane upon the concentration (1); 2,3 and 4 - concentration depolarization of tryptophane fluorescence with stimulation of respective wave length 302, 265 and 289 mmk.; b -polarization spectra of tryptophane fluorescence in concentrations, M: 1 - 10^{-2}; 2 - $7 \cdot 10^{-2}$; 3 - 10^{-1}; 4 - 1

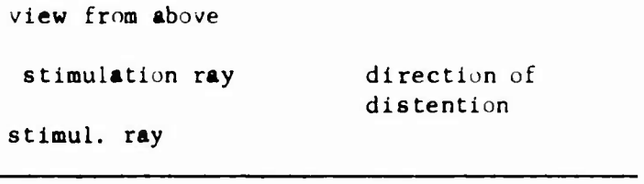
This portion of the fluorescence of the film may be 100°/o polarized, and its intensity is directly proportional to the relative quota of quanta, required for the absorption by oscillator ^1L_b as compared to the total absorption by both oscillators. In other words, the polarization spectrum obtained in this case represents the ratio $P_{lambda} = f\left(\frac{eta^1 L_b\ lambda}{eta^1 L_a + eta^1 L_b\ lambda}\right)$ and

actually is the relative spectrum of absorption of the negative oscillator.

Now, let us consider the results obtained experimentally. Fig. 16 shows a series of polarization spectra, obtained with oriented film of tryptophane under various conditions of stimulation and registration. One can see in the curve 4 of this figure that the degree of polarization for the region with waves shorter than 300 mmk. is far from being equal to zero, but rather has positive rates which change in a definite manner along the specter of absorption. Already the very fact of the presence of rates different from zero in the degree of polarization unequivocally indicates the presence of a second oscillator in the long wave band of absorption, oscillator ^1L_b, oriented to the oscillator ^1L_a under an angle, close to the right angle, since only thanks to such an oscillator the oriented tryptophane molecules are capable to absorb the light, in the given diagram of the experiment. The positive value of the degree of polarization indicates that the oscillator ^1L_b mostly does not luminesce itself, but through ^1L_a, for otherwise the rates(values) of the degree of polarization would have been negative. The character of the ratio p = f(lambda) shows that the maximum of absorption by t e electron passage ^1L_b is situated at 289 mmk, i.e. at the same point where is situated the minimum of polarization specter of fluorescnece afte absorption. At 300 mmk absorption ^1L_b is absent, and with this wave length the degeee of polarization of viscous solutions of tryptophane becomes the same as in the molecules with low segree of symmetry, approximated

with linear oscillator - 35 - 40°/o. The minimum of absorption of the negative oscillator is Also observed at 270 mmk which corresponds to the basic (main) maximum in the usual polarization spectrum of the isotope film (membrane(at 270 mmk.

Fig. 15. Diagram of experiments with polarization measuring of Absorption spectrum, conditioned by the oscillator ^1L_b



In a number of cases, one can even observe vibratory structure of the electron passage ^1L_b, observed in radiation of indol absorption and fluorescence spectra in neutral solvents and with the Shpol'skii effect. Of course, it is natural that the maximums of the vibratory structure of ^1L_b must appear in two minimums in the long wave portion of the polarization spectrum. Such polarization spectrum of fluorescence for absorption with two minimums in the long wave portion of the absorption band has been registered for N-glycyl-tryptophane in the 50°/o mixture of propylen-glyg l-water at the temperature of -70°C by Weber (1960) and for indol in sugar rock candy at room temperature, by us. The minimums of polarization spectrum were for glycyl-tryptophane situated at 286 and 293 mmk, for infol at 282 and 289 mmk.

In this manner, the polarization spectra for absorption, as well as the data on absorption and fluorescence spectra in various

solvents, confirm the presence of two electron transfers (trAnsfer, migration, passage, conversion?) ^1L_a and ^1L_b right term? in the long wave band of absorption. At the same time the electron passage ^1L_b A forms a narrow strip of absorption with maximum at 289 mmk., situated ßinside a wider band of absorption, conditioned by electron passage ^1L_a A. In this connection, the polarization spectrum of fluorescnce in absorption may be interpreted as follows:

1. The region of 300 and over mmk/absorbed is and radiated by the same oscillator ^1L_a. Here we have the maximum positive values of the degree of polarization, typical for molecules with the low degree of symmetry: p=38 - 40)/o.

2. Region 300 - 289 mmk - marked decrease in the degree of polarization with the minimum at 289 mmk: two mutually perpendicular oscillators ^1L_a and ^1L_b absorbs, mostly only one radiates, the ^1L_a. The drop in the degree of polarization towards the side of the short waves is dependent upon the gradual increase in the contribution of absorption by the negative oscillator. The minimum at 289 mmk corresponds to 0 - 0 passage (transfer) of ^1L_b, where the relative c ntributio of it to the total absorption is the maximum.

3. The increase in the degree of polarization 289 - 265 (270) mmk with the maximum at 265-270 mmk corresponds to the increase in relative absorption of the electron passage (transition) ^1L_a which has its maximum at 270 mmk.

4. Decrease in the degree of polAriztion 265-230 mmk reflects increase in relative absorption by oscillator ^1L_b, owing to the

intereffect with the oscillator ^1B_b in the absorption band ar 220 mmk.

Fig. 16. Tryptophane absorption spectrum on polyvinyl alcohol. 2, 7 - ratio $R_{fl.} = f(\text{lambda}_{\text{stimul.}})$ for tryptophane in isotrope film (membrane) of polyvinyl alcohol during stimulation with polarized light in vertical and horizontal directions respectively: 3, 4 - the same, as 2 and 7, but for tryptophane in anisotropiv film; 5, 6 - spontaneous polarization of fluorescence of tryptophane with vertical and horizontal position of stretched film (membrane), respectively.

Polarization spectra of fluorescence after emission for

In the section ,devoted to tryptophane fluorescence spectra were given proofs of the fact that not alone the long wave band of absorption, but the fluorescence spectrum itself is formed by two electron passages $^1L_a \rightarrow A$ And $^1L_b \rightarrow A$. At the same time, the polarization spectra of fluorescence for absorption show different spatial reciprocal orientation of the moments of passages ^1L_a And ^1L_b.

Therefore, one would expect that with monochromatic stimulation, the oscillator, forming the short wave portion of the fluorescence spectrum, would not correspond in its direction, to the oscillator, responsible for the remaining longer wave portion of the spectrum. In other words, formation of the fluorescence band by two different electron passages should have been apparent in essential differences in degree of polarization between the short wave and the remaining portion of the fluorescence band.

The shape of the polarization spectra of tryptophane fluorescence in the solid film of polyvinyl alcohol, completely confirms this prognosis (Fig. 13, curve 5). Actually, the degree of polarization retains its high and constant positive rates of the order of 25°/o throughout the entire band (strip) of fluorescence, with the exception of its shortest wave portion. From 315 mmk on there takes place gradual decrease in the dggree of polarization which in the region of 295 mmk already assumes low values -17°/o.

Everything is quite different in the polar solvent - glycerine polarized? in which fluorescence only occurs from one level of ^1L_a, and no drop in the degree of polarization accordingly is observed in the short wave portion of the fluorescence spectrum.

Similar polarization spectra for radiation were also obtained for indol, as in the ethyl alcohol at the temperature of 100°C (Zimmermann, Jopp, 1961). so also in polyvinyl alcohol at room temperature (Chernitskii, Konev, Bobrovich, 1963).

In indol, in polyvinyl alcohol, there are seen quite marked differences between the polarization of fluorescence in the main portion of the band and its short wave margin: the degree of polarization drops from 25 to 10°/o (Fig. 17, curve 5).

Apparently, the drop in the degree of polarization of fluorescence requires some explanation The invariability of the indol fluorescence spectrum (and that of tryptophane) in water, in polyvinyl alsohol and in hexane, with stimulation by various wave length within the limits of 250-300 mmk, indicates that, independenly of the ratio of the initial population of

the electron levels of ^1L_a AND ^1L_b, at the moment of emission (radiation) of light by the molecules, there is reached a certain equilibrium in the over-distribution of energy between the electron and the vibratory levels. At the same time, apparently, there occurs population (colonization) both of the level ^1L_a through the level ^1L_b, as well as the reverse process: this is the only explanation for the identity of the spectra of fluorescence, stimulated with the light of 296 mmk (initial population only of the ^1L_a level), to the spectra of fluorescence, stimulated with the light of 289 mmk (original population predominantly of the level ^1L_b). Although with "its own" absorption and "its own" emission of light with the participation of the ^1L_b level alone, polarization of fluorescence must have high positive values, peculiar within the limits of the common linear oscillator (40-50°/o), such a redistribution of energy results in that the polarization in the region of luminescence of ^1L_b passage may even become negative. Under the conditions pf equal quantum outputs of luminescens ^1L_a with its own stimulation and stimulation through the level ^1L_b, the degree of polarization in the first case will be positive in value, and in the second case - negative (or zero, depending upon the exact amount of the angle between the oscillators). Consequently, the degree of polarization of luminescence $^1L_b \rightarrow A$ can be determined only with quantitative ratio of absorbed quanta by the oscillators ^1L_a and ^1L_b with the given length of the wave. At the same time, it is obvious that the "positive" and "negative" oscillators should change places. In other words, in the registration of luminescence

^1L_b, in pure state without admixture of luminescence ^1L_a, the polarization fluorescence spectrum for absorption should be a mirror emage to the usual one: at the point of the maximum there would be situated minimums, and vice versa. Although such an experiment is impossible in its pure form, because of the mixed nature of luminescnce at any point of the fluorescence spectrum, nevertheless, one should expect changes in the shape of polarization spectrum for absorption with registration of fluorescence at the point lambda = 295 mmk. The curve 4 in Fig. 13 confirms these statements: in the former maximum of polarization spectrum at 270 mmk there is observed marked decrease in the degree of polarization, whereas in the former minumum at 289 mmk, the degree of polarization remains unchanged. It is not difficult to conceive that deparation of the registered luminescence from the admixture of ^1L_a should result in further changes in the shape of the spectra in this direction. The same picture is observed also in case of indol (Fig. 17, curve 4).

Fig. 17. Indol spectra in the film of polyvinyl alcohol at t=20°C.

1 - absorption; 2 - fluorescence; 3 - polarization with absorption after (registered 330 mmk); 4 - the same (registration 295 mmk); 5 - polarization fluorescence after radiation (stimulation 265 mmk); 12 - polarization spectrum of fluorescence, determining the relative contribution of "negative" oscillator to absorption; and at t = -196°C; 7 - phosphrescence; 8 - polarization phosphorescence spectrum after absorption (registration 480 mmk); 9 - polarization spectrum of phosphorescence after emission (radiation) (stimulation 265 mmk); 10 and 11 -spontaneous polarization

Fig. 17. cont. phosphorescence after emission (radiation) (stimulation 265 mmk) and absorption (registration 460 mmk), respectively; 6 ; ~~xxxxxxx~~ polarization spectrum of phosphorescence, determining relative contribution into absorption of "negative" oscillAtor (registration 460 mmk).

In this manner, the polarization spectra of stimulation of the short wave And long wave portions of the fluorescence spectrum are different, and this again is A proof of the property of the tryptophane molecules to illuminate the absorbed energy from two electron levels, the moments of the passage (transition) of which into the basic state are variously oriented in space.

Inasmuch as the oscillator ^1L_b in radiation only conditions an insignificant portion (5-10%) of fluorescence, then the main portion of the energy of stimulation must be passed on to the oscillator ^1L_a. The same is indicated by the coincidence of the spectra of stimulation of fluorescence of tryptophane and absorption spectrum. Such transfer is essentially equivalent ti extinction of its own fluorescence in ^1L_b and, therefore, cutting shorter the life span of the corresponding stimulated state. If it were possible to demonstrate the participation of two markedly different in their life span stimulated states in the formation of fluorescence band, that would be a strong argument for the possibility of fluorescence from two electron levels.

Thus, the question may be formulated as follows: is the tau of luorescence constant or not according to the emission spectrum?

To answer this question, Konev, Bobrovich, Chernitskii (1965) studies the shape of the polarization spectrum

studied the shape (form) of polarization spectrum of fluorescence after emission, in proportion to the increased concentration of tryptophane in the polyvinyl film. The concentration depolarization occurs differently in various points of the radiation spectrum. At the same time, throughout the entire main portion of the fluorescence band there is observed quite effective concentration depolarization, only sparing its shortest wave portion. Discrepancy in the ratio $R_{\text{la,bda emission}} = f(c)$ for the different portion of the fluorescence spectrum results in changing the shape of the polarization spectrum with greater concentrations; it becomes the oppoiste of that with small concentrations: instead of a drop in the degree of polarization in the short wave portion of the spectrum there is observed an increase (Fig. 18, a). A very weak concentration depolarization of fluorescence at thw short wave margin of the fluorescence spectrum shows that the life span of the stimulated state of ^1L_b is much shorter than ^1L_a. This confirms the participation of two single stimulated states of tryptophane.

Tryptophane Phosphorescence

Nature and Spectra of Phosphorescence

Tryptophane phosphorescence was first discovered in 1952 by Debye and Edwards and since then frequently studies by many authors. The characteristical peculiarities in the long wave luminescence of this aminoacid make it incontestable that its origin has the triplet-singlet transfer mechanism.

1. The phosphorescence spectrum is shifted into the long wave side, as compared with the fluorescence spectrum, i.e.

the phosphorescence level is situated within the scale of energy below that of fluorescence.

2. Tryptophane phosphorescence has a large life span (5.3 sec. after Longworth) which indicates strongly forbidden character of the triplet-singlet passage (transfer) (T S).

3. For the triplet luminescence the monomolecular law of exponential extinction should be observed. This was actually observed by Debye and Edwards, 19520, as well as by Steel and Szent-Gyorgyi, 1957, in water-glucose frozen solutions of tryptophane.

4. The intensity of phosphorescence is increased with addition of heavy atoms of potassium iodide (Vladimirov and Li and Chin-go, 1962)/lithium bromide (Konev, Bobrovich, 1964), which facilitate conversion of the spin by their field.

5. The process of tryptophane phosphorescence practically requires no energy of activation (from the data of Still and Szent-Gyorgyi, 1957), E = 0.076 kcal)

6 Tryptophane phorsphorescence has a negative value of the degree of polarization with stimulation along the entire absorption spectrum, ~~with-the-basic-state-of-absorption-spectrum, as-shown-by-Grossweiner-with-the-method-of-flash-photolysis;~~ i.e the phosphoresnce oscillator is perpendicular to the fluorescence oscillator (Chernitskii, Konev, Bobrovich, 1963).

7. The tryptophane molecules in phosphorescent (triplet) state have an absorption spectrum, shifted into the long wAve side as compared with the basic state, as shown by Grossweiner, 1956, 1959 with the method of flash photolysis.

8. Tryptophane and indol possess alpha-=h sphorescence in solution (Barenboim, 1962), in the film of polyvinyl alcohol (Konev, Chernistkii, 1964) and in crystals (Chernitskii, Knev, 1964).

9. Concentration of non coupled (unpaired) electrons of tryptophane molecules in triplet state should decrease after the flare up of light, according to the same law, as the phosphorescence. As a matter of fact, Smaller, 1962, for indol, and Ptak and Douzou, 1963, for tryptophane have registered some photoinducted signals EPR which they ascribed to triplet states.

According to Gribova, 1964, in ethanol solution of tryptophane at 77°K nad in illumination by integral light of the lamp SVDSh-1000 there was observed the EPR tryptophane signal with the extinction time about 3 sec. Before the absorption of the quantum of tryptophane molecule, which later are to emit fluorescence or phosphorescence light, in no way differ one from the other: the spectra of phosphorescence stimulation coincide with the spectra of fluorescence stimulation (Vladimirov, Litvin, 1960).

Fig. 18. Polarization spectra of fluorescence after emission: a - polyvinyl alcohol films, activated 1, 2 and 3 - tryptophane in the concentrations of $5 \cdot 10^{-1}$, $5 \cdot 10^{-2}$, $5 \cdot 10^{-3}$ g/g respectively; 4 - glycyl-tryptophane in concentration $5 \cdot 10^{-3}$ g/g; b - proteins; 1 and 2 water solutions of hemoglobin and trypsinogen and trypsin; wool keratin, (fibers not oriented); 4 - wool keratin, extracted twice with water vapor and oriented parallel to the

cont/

Fig. 18 cont. electrical vector of the stimulated light. In all cases, fluorescence was stimulated at 265 mmk.

Freed and Salmre were first to obtain well solved phosphorescence spectra in 1958 for methanol-ethanol (9:1) tryptophane solution, cooled down to the temperature of liquid nitrogen, for indol, indolyl-acetic acid and tryptophane with the use of phosphoscope with solution time $5 \cdot 10^{-3}$ sec. These authors noted definite maximums of tryptophane phosphorescence, situated at 408, 438 and 460 mmk. For the water-salt solutions Vladimirov and Litvinm for prolonged past-radiation (1960), Vladimirov and Li Chin-go (1962) and Burstein (1964), for low temperature luminescence (without the use of phosphoscope) registered maximums at 406, 432 and 456 kkm. and the arm ("shoulder", lever?) at 470-480 mmk/ The phosphorescence spectrum, as also that of fluorescence, is conditioned only by the indol ring proper, without any noticeable participation of the lateral groupls; this is reflected in the nearness of the phospgorescence spectra of indol, tryyotophane and other derivatives (Freed and Salmre, 1958; Chernitskii, Konev, and Bobrovich, 1963), Not dwelling at length on the details of phosphorescence spectra, let us mention its main peculiariry (Fig. 13, curve 7) - that of clearcut structure in constrast to lack of structure under the same condition in the fluorescence spectra, in spite of the fact in both spectra there is apparent the same system of energy [illegible] levels of the lower state and there are no [illegible] noticeable differences between the stimulation spectra of these two forms of luminescence (Vladimirov, Litvin, 1960).

These spectral peculiarities led Vladimirov and Li Chin-go (1962) and Vladimirov (1964) to assume that phosphorescence is produced from the unbalanced, little probable vibratory cpnfiguration of the tryptophane molecule. The whole chain of events, leading to the act of phosphorescence, according to these authors, has the following appearance: S_0 S_1 T_1 A S_0, where the passage S_0 S_1 coreesponds to absorption, S_1 S_0 to fluorescence, T S_0 to phosphorescence, and A to the unbalanced vibratory state. Therefore, phosphorescence is associated not alone with the rotation of the spin revolving but likewise with the changes in the molecule configuration. With the latter, these authors explain the great duration of tryptophane phosphorescence (5 sec.). This interpretation appears to us not quite satisfactory. First of all, the greater duration of phsosphorescence is caused not by configurational reconstructions, but by the probable revolutions (turning) of the spin, the spin-orbital intereffect. In the presence of heavy atoms (litium bromide) lithium, with unchanged spectrum of phosphorescence, its life span is decreased several times (Konev, Chernitskii, 1964). Moreover, well built structure of phosphorescence spectrum, apparentiy, does not appear, as result of the appearance of little probable configuration of the molecule, but most likely precisely reflects the usual state of the molecule, not complicated by different intereffects. The cause of the discrepansy (non-conformity) of the spectra of fluorescence and phosphorescence may be seen in case the problem is solved in the reverse manner: precisely, owing to the fact that the fluorescence spectrum is determined more

more not so much by the inner energy properties of the molecule, but rather the outside medium (environment) and there is no resemblanee in the phosphorescence and fluorescence spectra. In indol, in non polar (polarized?) solvents (hexane, cyclohexane), as well as in tryptophane residues in the composition of amylase crystals at the temperature of 77°K, i.e. under conditions which are favorable for the appearance of molecular spectrum, the position of the maximums of phosphorescence in the scale of frequencies changes litte, while in the fluorescence spectrum there appear some frequencies, typical for the phosphorescence spectrum. The frequency symmetry between the molecular spectrum of phsophorescence and fluorescence, on the one hand, and the tryptophane phosphorescence spectrum in the polar solvents, on the other hand, shows that in the polar solvents the molecules in the triplet state are less energetically distorted, than in the singlet state. Against the Vladimirov's diagram speak indirectly the independence of the spectra and the intensity of tryptophane phosp orescence and that of indol from the wave length of the stimulating light, in case of monochromatic stimulation within the limits of 240-313 mmk. Consequently, the molecules with different store of vibratory energy indistinguishable one from the other, so far as their capacity to phosphorescence and its spectral composition are concerned. A number of circumstances lead us to believe that the greatest importance in the changing of probabilities of triplet-singlet conversions in tryptophane molecule belogs to hydrogen in the iminogroup. As a matter of fact, indol in non polar solvet - hexane - is almost

completely deprived of fluorescence and possesses intensive phosphorescence (free NH-group), indol and tryptophane in alcohols (methyl, ethyl, butyl, ethene-glycol) and in water-salt solutions have average and identical values of fluorescence intensity (NH-group, forming hydrogen bond) and, finally, in 0.1 M NaOH, where occurs complete ionization of the iminogroup, phosphorescence disappears Almost totally. In all cases, the form of the stimulation spectra and phosphorescence spectra suggests that we are dealing with monometrical centers of luminescence. Therefore, gradual increase in the distance between hydrogen and nitrogen in the iminogroup is accompanied by weakening of relative intensity of phosphorescence. The role of the iminogroups in the determination of relative probability of singlet-triplet passages (trAnsfers? conversions?) is clearly seen in the examples of tryptophane in crystal state. (because of its low intensity)
As we know, it is possible to observe phosphorescence/of the tryptophanse crystall only after separating it from more intensive fluorescence with the aid of a phosphoscope (Bobrovish, Konev, 1964). In crystal state, tryptophane differs sharply from tryptophane in solution, not alone by its low intensity phosphorescence, but likewise by the fact that its specter is shifted into the long wave side up to 500 mmk. and loses its structure. A similar picture is obtained also in concentrated aequeous solutions of tryptophane: in addition, admixture of substances which bind hydrogen in the iminogroup and thereby preventing association (formaldehyd), brings about restoration of the usual phosphorescence of the monometric tryptophane.

Apparently, tryptophane in the crystalline network (framework) is in the form of dimers, formed with participation of hydrogen of the iminogroup. In favor of thj is is the displacement of the frequencies of NH vibrations in the crystal (crystalline) specimens of tryptophane and indol before the position on the scale of the frequencies of infrared spectrum, typical for the mentioned hydrogen-bound N...H - N group. Moreover, jusding by the spectra of stimulation, the absorption spectrum of the "crystalline" molecules of tryptophane is shifted by 3-4 mmk. into the long wave side, as compared to the monometric forms (up to 285 mmk).

Alpha-Phosphorescence of Tryptophane

Barenboim, 1962, succeeded in registering non spectrally, the alpha-phosphorescence of tryptophane. This luminescnce, as we know, appears as a result of thermoactivated return with rotation of the electron spin from the triplet level to the signlet with subsequent radiation, following the mechanism of usual fluorescence. As a consequence of this, in the spectrum, alpha-phosphorescence should coincide with fluorescence, have a considerable life span and become increased within certain limits in proportion to rising temperature. The last named two circumstances were actually observed in the mentioned work. The spectra of alpha-phosphorescence of indol and tryptophane in the polyvinyl alcohol film at the temperature of liquid nitrogen have been studied in our laboratory and were found to be similar to the fluorescence spectra under the same conditions (Fig. 19). However, with the time permissible with phosphoroscope at 10^{-2} - 10^{-3} sec.

the intensity of alpha-phosphorescence was 4 times weaker than the beta-phosphorescence. Intensive alpha-phosphorescence with very fine structure, has been registered only for indol crystals (Chernitskii, Konev, Fig. 20).

Fig 19. Indol alpha-phosphorescence spectra (1) and tryptophane alpha-phosporescence spectra (2) in films of polyvinyl alcohol at - 196°C. Permissible time of phosphoroscope 10^{-3} sec.

-Fig 20. Luminescence spectra of indol crystals at room temperature (1) and at t= -196°C (2).

Polarization Phosphorescence Spectra for Absorption

Polarization spectra of tryptophane phosphorescence after absoption were measured by the author in 1963 together with Chernitskii and Bobrovich. The integral polarization spectrum, obtained with registration of tryptophane phosphorescence in polyvinyl alcohol at the temperature of liquid nitrogen is represented in Fig. 13, curve 8. Rather high negative values of the degree of polarization of phosphorescence throughout the entire Absorption spectrum indicate that the oscillator of phosphorescence makes an angle, close to a right angle, both with the oscillator ^1L_a and the oscillator ^1L_b, i.e the oscillator of phosphorescence is perpendicular to the plane of the indol ring Similar orientation in space have oscillAtors, corresponding to each one of the main three maximums of phosphorescence spectrum - 410, 438 and 460 mmk., since the polarization spectra of

phosphorescence after absorption are identical at these points in other words, all the three well solved maximums of phosphorescence should be ascribed to one and the same electronpAssage.

A slight decrease in the negative values of the degeee of polarization of phosphorescence at 289 mmk. indicates that the oscillator of phosphorescence is not strictly perpendicular to the oscillator of the electron passage $^1L_b \rightarrow A$, but has a small component in its direction. The smallness of this gap (1÷2 °/o as compared to 6 - 7 °/o in the polarized spectrum of fluorescence) indicates that the angle between the phosphorescence oscillator and absorption oscillator ^1L_b is still very little different from a right angle. The existence of "negative" oscillator in the long wave band of absorption finds additional confirmation also in the experiments with the induced polarization of phosphorescence of anizotropic films of tryptophane and indol in the passing light. With coincidence of the direction of distention of the film with the direction of vibrations of the polarized light of stimulation P_{phosph} is - 30°/o, with mutually perpendicular direction (90° turn of polarizator or the film), the P of phosphorescence suddenly assumes positive values +8°/o. In the isotrope film of the same concentration, in both cases, P = -13°/o Such a change in the symbol of the degree of polarization indicates rhe presence in the molecules of anisotrope films of absorption oscillators, predominantly perpendicularly directed to distention oscillators of absorption ^1L_b, i.e "negative" oscillators.

The anisotrope films of polyvinyl alcohol, activated with

tryptophane, permit the use of a methods, developed in our laboratory, for demonstrating the spectrum of absorption of the negative oscillator ^1L_b in the case, if its absorption is actively used for the atimulation of phosphorescence. The spectrum which reflects the ratio

$$P_{phosph} = f\left(\frac{eta^1 L_{b\ lambda}}{eta^1 L_{a\ lambda} + eta^1 L_{b\ lambda}}\right).$$

is obtained with a mthod similar to the one described in the section onf the "Polarization Spectra of Tryptophane Fluorescence". Only in this case, the negative oscillator of absorption will pass on to the oscillator the absorbed energy, to the scillator, situated transversely across the direction of the distension, i.e. horizontally. As a consequence of this, on the fully depolarized phosphorescence in the chaotically arranged portion of hthe molecules there will be superimposed the negatively polarized phosphorescence, conditioned by the absorption of the oscillator ^1L_b in the oriented portion of the mlecule. However, the spectrum of absorption of the negative oscillator must remain unchanged, both when it is found with the aid of polarized fluorescence and with the aid pf polarized phosphorescence.

Experiments fully substantiate the above line of thinking, once more proving, in this manner, the complex natuee of the long wave band of absorption of tryptophane and indol. As one can see in curve 12 in Fig. 17, the polarization phosphorescence spectru, of absorption is actually completely situated in the region of the negative values of polarization and has maximum

negative values of the degree of polarization at 289 mmk. At 300 mmk, where the oscillator ^1L_b does not absorb, the degree of polarization assumes zero values.

Polarization Spectra of Phosphorescence after Emission

The polarization spectra of tryptophane and indol phosphorescence, after emission, studied in the same work with the polarized spectra of phsophorescence after absorption (Chernitskii, Konev, Bobrobich, 1963) are represented in Fig.13 and 17, curve 9. The common peculiarity of these spectra consists of a certain increase of negative values of the degree of polarization to the short wave margin of the phosphorescence band. In the maximum 410 mmk P_{phosph} attains the value $-18^{o}/o$. On the other, the long wave, margin of the phosphorescence band the values of polarization degree are lesser, around $-10^{o}/o$. Anpther characteristical peculiarity of the polarization spectra is the presence of the vibratory structure, and the values of the degree of polarization have maximum negative values in the maximums of phosphorescence and minimal in the minimums. Such a vibratory structure of the polarization spectrum has been registered not only for tryptophane, but also for indol and indolylpropionic acid. The elements of the fine structure of the polarization spectra of phosphorescence are particularly well manifested in the polarization spectra of spontaneous polarization of phosphorescence in the anisotrope films (Fig. 13 and 17, curve 10). Such a structure may be the consequence of superposition of two vibratory series of one electron passage, as

this has been accepted by Zimmermann.

Oscillatory Model of Tryptophane Molecule

As mentioned before, the question of the orientation of the oscillators with reference to each other may be solved, on the basis of analysis of the polarization spectra of luorescence after absorption and emission. The stability of the values of the degree of polarization fluorescence of tryptophane and indol in the region of 330-360 mmk is an indication that in this region radiation is conditioned by one oscillator. From comparisoon of the polarization spectrum of fluorescence with the spectrum of absorption of indol it may be concluded that this oscillator must be ^1L_a (the maximum rates of the degree of polarization correspond to those zones in the sbsroption spectrum where the contribution of the electron passage ^1L_a is the greatest). From the form (shape) fo the polarization spectrum of fluorescence after absorption it follows that the second oscillator of the long wave band of Absorption is ^1L_b, since it corresponds to the gap in the polarization spectrum at 289 mmk. and is directed at an angle close to 90° with reference to the oscillator ^1L_a (marked overlapping of the bands ^1L_a And ^1L_b in the absorption spectra of indol and its derivatives in the polar solvents does not permit to solve this question definitively, whether the angle between the oscillators is 90° or less). The negative values of the degree of polarization of fluorescence in the area of the second band of absorption 220 mmk and the negative symbol of its dichoi dichroism indicate that the oscillator of this band is oriented perpendicular to the oscillator

^1L_a. The negative values of the degree of polarization of phosphorescence, with stimulation at all points of the absorption spectrum denote orientation of the oscillator of phsphorescence perpendicular to the absorption oscillators and fluorescence oscillators.

Fig/21. Dependence of P_{cn} of tryptophane fluorescence upon the azimuth of the film, t=20°C.

A number of proofs of the correctness of the above conceptions on the mutual orientation of absorption and emission oscillators may be derived from experiments with oriented tryptophane molecules, and those of indol, and indolyl-propionic acid. Orientation of the molecules was accomplished with mechanical stretching of the filmsd/ of polyvinyl alcohol, activated with indol or tryptophane. It was necessary to solve the problem, how the fluorescence oscillator becomes oriented with reference to the direction of the stretching of the film. For that purpose, the ratio of the degree of spontaneous polarization of fluorescence upon the azimuth of the film was determined, i. e. the angle between the vertical direction and the direction of the stretching of the film The ratio $P_{fl} = f(\varphi$ were obtained for tryptophane (Fig. 21), indol and acethyl-tryptophane. From the illustration one can see that the maximum positiove values are observed with $\varphi = 0°$, and the maximum negative with $\varphi = 90°$. With $\varphi = 45°$ there are found zero valuesfor the degree of polarization. The same ratio are found also for acethyl-tryptophane and indol. Inasmuch as, in these experiments, fluorescence was registered

in the regions of 330-360 mmk., where polarization remained c . ant, and since the maximum positive values were shown for this fluorescence wherever the relative contribution of the oscillator ^1L_a is maximum ($^{265}_{285}$, 300 mmk), all this signifies that we are dealing, first, with the oscillator ^1L_a in fluorescence, and, second, this oscillator is oriented along the direction of the distention of the film. The same films assume maximum values of dichroic ration with = 0°. This concurs with the general rule for all organic molecules, in which the oscillator of the long wave absorption corresponds with the fluorescence oscillator.

Therefore, tryptophane molecules and indol molecules are oriented inthe film in such a manner that the oscillator ^1L_a in absorption and in emission (radiation) is situated long the direction of stretching. It still remains to be determined, how the tryptophane molecules proper are oriented in the film so as to bind this oscillator to the molecular skeleton. At first glance, it appears the most natural to assume that the stream (flow) of the threads (fibers?) of polymer sliding along each other orients the tryptophane residues with the long axis along the tension. In this cAse, the substitutes which lengthen the molecule would be facilitating orientation in the film. However, experiments revealed the opposite picture. The amount of the spontaneous =plarization of fluorescence films, stretched an identical number of times (five-times tension) was found to be greater in indol (20°/o) than in its derivatives (in tryptophane, for example, pnly 13°/o). The films, activated with indol, also possessed higher rates of spontaneous polarization phosphorescence and dichroism.

phosphrescence and dichroism. Hence, indol and rryptophane molecules are oriented along the tension with their shorter axis, and the lateral chain of tryptophane interferes with the rocess of orientation. This is xxx confirmed also by the property pf hydrogen in the iminogroup to form hydrogen bonds and this inevitably must make more difficult the orientation of the longer axis of the molecule along the tension and, conversely, it must promote the orientation with the shorter axis.

Thus, the oscillator ^1L_a is precisely situated along the shorter axis of the molecule; from the above described, this axis is also oriented along the tension of the film. On the other hand, this conclusion concurs well with those made earlier, based on the different shift (displacement(of the absorption bands ^1L_a and ^1L_b during the passage (change, transfer) from hexane to alcohol solutions: the oscillator ^1L_b is directed along the long axis of the molecule, the oscillator ^1L_a, along the shorter axis.

It should be noted that the mentioned (position) arrangement of the oscillator, found from experimental data, is in full Agreement with the theoretical predictions, based on the simplified model of the perimeter. For naphthalene, orientation ^1L_a passage along the shorter molecular axis was assumed, and ^1L_b, along the longer axis (Platt, 1949). Taking into consideration that both naphthalene and indol are iso-pi-electronic svstems, and their spectral characteristics are close, Platt, 1951, assumed they have identical orientation of oscillators of electron passages (trAnsfers). Later, Zimmermann and Jopp adopted Platt's

point of view (1961). Weber held similar opinion, as reported by Shifrin, 1964.

However, in indol and tryptophane the oscillator ^1L_a is situated not strictly along the shorter axis of the molecule, but at a certain acute angle to it: the angle between the oscillators ^1L_a and ^1L_b is less than 90°. This allowance should be made, based on the form (shape) of of polarized spectra of fluorescence after absorption for indol and its derivatives. From the indol absorption spectra in hexabe it follows that the molar extinction of the band ^1L_a and ^1L_b are just about even. Judging from the form of the spectra of absorption in alchhol solutions and solid films of polyvinyl alcohol, in the region of 289 mmk the contribution to absorption of the oscillator ^1L_b is not lesser that ^1L_a. With their perpendicular reciprocal orientation, this should be accompanied by complete depolarization of fluorescence at this point; however, this does not really occur. Moreover, since the absorption oscillators ^1L_a and ^1L_b are situated in the plane of the molecule and the oscillator ^1L_a is oriented along the tension (stretching) of the film (membrane), then under the conditions of mutual perpendicular stretching of the oscillators, the absorption spectra would be different on measuring in vertical and horizontal polarized light. The course of the dichroism spectra in this case should correspond to the course of polarization spectrum, since this is observed inthe majority of dyestuffs (pigments) (Feofilov, 1960).

Actually, in indol there have been found to minimums in the dichroism spectra at 282 and 290 mmk (Fig. 22). However,

the absolute rate of these minimums is considerably smaller, than in the polarized spectra of fluorescence. The non-coincidence of the polarization spectrum of fluorescence with the dichroism spectrum may be understood, if we assume that the oscillator ^1L_b is oriented at an angle, somewhat smaller than 90° to the direction of the oscillator ^1L_a.

Fig. 22. Dichroism of anisotrope film of polyvinyl alcohol, activated with indol (1), And its absorption spectrum (2).

In this manner, certain data indicate that the angle between the oscillators ^1L_s and ^1L_b for indol and its derivatives is less than 90° and is situated within the limits of $45\text{-}90^\circ$.

Fig 23 Polarization spectra of phosphorescence after emission of anisotrope films of polyvinyl alcohol, activated with indol (measurements at the "gap"); 6 - direction of stretching of the film coincides with the direction of electrical vector of the stimulating light; 5 - the film is turned in its plane at 90 and the ratio of the spectra of spontaneous polarization of phosphorescence for the same film and tha axiuhu azimuth of the film: 1 - φ 90; 2 - φ 70; 3 - φ 20°; 4 φ $=0^\circ$.

After tying the oscillator ^1L_b to the long wave axis of the indol molecule, the tying of oscillator ^3L_a, corresponding to the passage from the metastable to the basic state, could only be made based on the experiments with thw study of polarized spectra of siotrppe films. Based on the shApe of the polarization

spectrum of phosphorescence, obtained in our laboratory (Fig. 17, curve 8), it may be concluded that the oscillator of phosphorescence is essentially perpendicular to the absorption oscillators ^1L_a and ^1L_b. This is in addition confirmed by the anisotrope films. The perpendiculat position of the phosphorescence oscillator to the oscillator ^1L_a is proven in experiments on the study of the ratio of the extent of the spontaane us polarization adn the azimuth of the film. The maximum rates in the degree of polarization at =90° definitely convince (us) that the phosphorescence oscillator is perpendicular to the oscillator ^1L_a (Fig. 23).

A small gap in the region of 289 mmk and peak (point) at 296 mmk in the polarization spectra of phosphorescence of isotrope films indicates that the phosphorescence oscillator is not strictly perpendicular to the other oscillator - oscillator ^1L_b. This conclusion is further confirmed by the fact that with stimulation of phosphorescence with the mercury line 265 mmk the polarization spectrum of indol phosphorescence after emission represents a nearly straight line, whereas with stimulation 280 mmk the spectrum has a distinct structure, apparently conditioned with the superposition of two series of electron vibratory bands, polarized in mutually perpendicular directions. Naturally, with the aid of the line 280 mmk, there is stimulated relatively greater portion of the oscillators ^1L_b which have a corresponding component (in direction) in phosphorescence, and this results in lessening of the degree of polarization in the corresponding points of the spectrum. With stimulation,

the, only of the oscillator ^1L_a (or to a greater extent, ^1L_a), the oscillators of both β vibratory series of the band of phosphorescnce are oriented to it perpendicularly, and this results in constancyof the negative values of the degree of polarization throughout the entire phosphorescance spectrum

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Fig. 24 Polarization spectra of phosphorescence of indol after emission in the films of polyvinyl alcohol with stimulation lambda = 280 mmk (1) and lambda = 265 mmk (2).

Fig. 25 Diagram of electron vibratory levels of indol and tryptophane molecules and orientation of corresponding oscillators with reference to the "skeleton" of the molecule.

Similar proportions (ratio) are also observed experimentally (Fig. 24).

Finally, a few words on the reasons which permit us to identify the phosphoresce ce state of tryptophane as ^3L_a. As we know, based on the law of selectivity of the partially solved spin-orbital intereffect T-S of the passage and the degree of polarization of phosphorescence for stimularion in the maximum ^1L_a and ^1L_b, it is possible to establish the symmetry of the phosphorescent staye (William, 1959). Theoretical calculations lead us to the conclusion that the lowest truplet state of the aromatic hydrocarbons must be ^3L_a (ϕ Pariser, 1956). This theory is well confirmed experimentally for such a compund as pheantren (Azumi, McClynn, 1962).

In the case of tryptophane and indol, there is additional

confirmation of the nature of T-S passage, in the partial coincidence of phosphorescence and ^1L_a-fluorescence of the amilase and indol crystals in cyclohexane.

The above statements may be generalized as follows (Fig. 25). The oscillator ^1L_b, corresponding to the electron passage $^1L_b \rightarrow A$ with the maximum at 289 mmk in the long wave band of absorption and the electron passage $^1L_b \rightarrow A$ in the short wave portion of the /M/ fluorescence band, is oriented in the plane of the molecule along the long axis of it. The oscillator ^1L_a, corresponding to $^1L_a \leftarrow A$ the electron passage/, with the maximum absorption 266 mmk (in hexane) and maximum at 272 mmk in polar solvents and electron passage $^1L_a \rightarrow A$ in emission (fully conditioning fluorescence in aqueous solutions), is situated in the plane of the molecule under an angle lesser than 90° with reference to the oscillator ^1L_b. Between the electron levels levels ^1L_a and ^1L_b, both participating in fluorescence, there exists effective re-distribution of energy. The oscillator ^1B_b, correpponding to the electron passage ^1B_b A, with maximum at 220 mmk in absorption, is situated at an angle close to 90° with reference to the oscillator ^1L_a. The oscillator of phosporescence ^3L_a is oriented perpendicular to the plane of the molecule, having a small component along the longer molecular axis, i.e. it is perpendicular to the pscillator ^1L_a, but is not strictly perpendicular to the oscillator ^1L_b. An unusual conclusion, derived from the study if theluminescent properties of tryptophane, is the conclusion on the possible fluorescence from two electron levels, the conclusion on the existence of two singlet electron-stimulated states of this

aminoacid. As we know, the majority of organic compounds have characteristic appearance of radiation passages from the zero vibratory sublevel of the lower electron level, independent of the site of the original (initial) stimulation on the scale of energy. Concept of the possibility of rAdiation of quanta of fluorescence from two levels by the indol molecules is now elaborated by many authors: Zimmermann et al (Zimmermann and Geisenfelder, 1961; Schuett and Zimmermann, 1963; Zimmerman and Joop, 1961).

Apparently, indol and its derivatives could not be correctly considered as the only compounds capable of fluorescens from two levels. Fluorescence fromthe second level has been found in azulene (Zimmermann and Joop, 1960), and from the first and the second levels, in paradisubstituted derivativesx of benzol(Lippert, Luder and Boos, 1962; Roem 1961), r-cyano-dimethyl-aniline (Lippert, 1962).

Tyrosine

Tyrosine fluorescence (Fig. 20) hasb been studied by many authors both in aquesous solutions (Bowman et al, 1955; Vladimirov and Konev, 1957; Cowgill, 1963; Teale and Weber, 1957), and in crystals (Brumberg, 1956; Vladimivor, 1961). The maximum of fluorescence band of aqueous solutions is situated at 303 mmk. With freezing of aqueous solutions, fluorescence maximum is shifted into the short wave side and is situated at 298 mmk (Vladimirov, Li Chin-Go, 1962). Tyrosine crystals possess the same spectrum of fluorescence, as the aqueous solution (Barskii, Brumberg, 1958).

Lehrer and Fasman, 1964, reported on "ekzaimeric" (not in any dictionary, not a Russian word) fluorescence of polytyrosine at 420 kkm.

Fig. 26. Tyrosine, aqueous solution.
1 - absorption spectrum (solid line) and spectrum of fluorescence stimulation (dotted); 2 - fluorescence spectrum (Teale and Weber, 1957); 3 - polarization spectrum of fluorescence after absorption (propylene-glycol, stimulation with non polarized light, Weber, 1960); 4 - luminescence spectrum at t= -140°C (Vladimirov, Burstein, 1960); 5 and 6 - luminescence spectra respectively in 0.1 M glycocol and 0.1 H NaOH at t= 77°K (Vladimirov, Li-Chin-go, 1962)

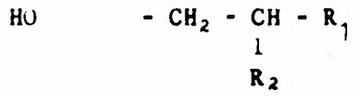
The fluorescence stimulation spectrum coincides with the absorption spectrum, i.e. has its maximum at 275 and 222 mmk (Teale and Weber, 1957). According to the data of the same authors, the quantum output at any length waves of stimulation is identical equal to 0.21..

Cowgill, 1963, apparently, made no correction for the spectral sensitivity of the apparatus; still he mentioned coincidence in the position of fluorescence maximums of tyrosine and m-creosol, OH-benzyl alcohol; phenol, l-tyrosil-glycerine, glycyl-l-tyrosine, tyramine, glycyl-l-tyrosil-glycynamide, leucyl-l-tyrosine (320 mmk). spellings?
Hence, it follows that the position of the maximums of fluorescence is determined exclusively by the phenol nucleus without any effect from the substitutions. Things are quite different with the quantum output of fluorescence. Lateral groupings are capable of exerting marked influence on the absolute amount

of output. Especially strongly extinguishes fluorescence the non ionized carboxyl grouping (Table 3).

Table 3

Effect of Lateral Substitutions on the Quantum Output of Fluorescence of Tyrosine Derivatives



Compound	R_1	R_2	φ
Tyrosine	$-COO^-$	$-\overset{+}{N}H_3$	0.21
Tyramine	$-H$	$-\overset{+}{N}H_3$	0.185
Leucyl-tyrosine	$-COO^-$	^+_3HN-CH-CO-NH- (leuc.)	0.103
Tyrosyl#glycin	$-CO-NH-CH_2-COO^-$	$-\overset{+}{N}H_3$	0.074
Glycyl-tyrosine	$-COO^-$	^+_3HN#CH_2-CO-NH-	0.070
Tyrosine	$-COOH$	$-\overset{+}{N}H_3$	0.056
Glycyl-tyrosyl-glycylamide	$-CO-NH-CH_2-CO-NH_2$	^+_3HN-CH_2-CO-NH-	0.035
Glycyl-tryosine	$-COOH$	^+_3HN-CH_2-CO-NH^-	0.027

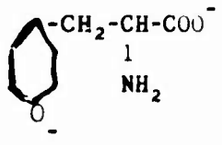
The presence in the composition of tyrosine molecule of some dissociating ionogen groups also determines the dependence of the quantum output of fluorescence upon the pH of the medium (Fig. 9). The dependence of the quantum output of fluorescence upon the pH has been studied by White, 1958, Vladimirov and Li Chin-go, 1962, Cowgill, 1963. The decrease in the quantum output in the region of low pH, starting with 4.0, is caused by the change of the carboxyl group from the ionized state into the non ionized state: $R\text{-}COO^- \rightarrow R - COOH$.

Tyrosine with non ioninged carboxyl groups has a quantum

iutput 0.056 (Cowgill, 1963), pF of this passage (transfer), measured by the intensity of luminescence, coincides with pK, measured during amperometrical titration - 2.2, after Cowgill, and 2.45 after White.

The area of the second drop in quantum output of tryssine fluorescence is associated with dissociation of the phenol hydroxyl and has pK = 9.7, after White. White,. Cowgill, Vladimirov and Li Chin-go believed that the dissociated form of tyrosine is deprived of the capacity to luminesce.

However, Cornog and Adams, 1963, observed a weak fluorescence of tyrosine in 0.12 N NaOH with the maximum at 345 ± 5 mmk, with the quantum output of the order of 0.01, and they ascribed it to the phenol ion of tyrosine. This concurs with the earlier data of Longin, 1959, on the fluorescence of alkaline tyrosine in the region of 400 mmk. Consequently, along with extinction of the fluorescence of tyrosine in the non ionized form there appears a weak fluorescence of ion



Corresponding to the latter form there is, apparently, the maximum of low temperature fluorescence (77°K) of tyrosine in 0.1 N NaOH at 320 mmk and the maximum of phosphorescence at 410 mmk (Vladimirov, Li Chin-go, 1962).

The polarization spectra of tryosine fluorescence have been studied by Weber, 1960. According to his findings, in propylene glycol at the temperature of -70°C, tyrosine and cresol have

identical polariztion spectra (Fig. 26) which show that the long wave band of absorption is formed by one electron passage. The absolute values of the degree of polarization withn the limits of the absorption band 275 mmk 0.21 with recounting into the linear polarized stimulatuon according to the formula $P = \frac{2P_H}{P_H + 1}$ assume the rates 0.35, i.e. such rates which are characteristical for the non symmetrical molecules and molecules with the symmetry of the second order. As we know, the flat (plane) molecule with the axis of symmetry of the third order and above cannot have borderline polarization over 1/7, i.e. a rate, obtained with the cAlculation of the classical model of the rotating linear oscillator and with thequantum mechanical calculation of the process of luminescence of two-atom molecules (Feofilov, 1959). Actually, the flat benzol molecule which hAs the axis with symmetry of the 6th order, perpendicular to the plane of the molecule, (symmetry D_{6_h}), after Feofilov, has low values of the degree of polarization: 7.7°/. Phenol likewise has low rates of polarization. Hence, only the addition of alanine residue in the para-position is accompanied by marked changes in symmetry of the electron cloud in the benzol ring with transition from the flat to the linear sciliator. It is characteristical that shifts of the levels in the energy scale, then, do not occur. The oscillator, corresponding to the second electron passage in the band 222 mmk is oriented at a right angle to the long wave oscillator of absorption, inas much as Weber observed in the region of 230 mmk negative values of the degree of polarisation.

The tyrosine phosphorescence was first observed by Debye

and Edwards, 1952. The tyrosine phosphorescence spectrum, registered vy St eel and Szent Gyorgyi, had a wide maximum in the region of 370-410 mmk (Steel and Czent Gyorgyi, 1958). Vladimirov and Litvin, 1960, Vladimirov and Burstein, 1960, observed, in the tyrosine phesphorescence spectrum at -100 °C the maximum at 387 mmk and a weak shoulder (arm) 410-420 mmk.

The data of the Soviet authors were confirmed by Longworth, 1961, who obtained position of phosphorescence maximum at 388 mmk. Under certain conditions, it is possible to convert the non structured band of phosphorescence of tyrosine into a spectrum, well solved into separate vibratory maximums. Certain elements in the structure of phosphorescence apprar in strongly alkaline medium (Vladimirov, Li Chin-go, 1962). In the 8 M litium iodide, which facilitates the singlet-triplet conversion, there are registered sharp maximums of phosphorescence 354, 366, 376, 387, 397, and 412 mmk (Konev, Bobrovich, 1956).

The span of life of tyrosine phosphorescence tau in neutral and acid medium is 3 sec., and in the alkaline medium, 0.9 sec. (Debye and Edwards, 1952)$ More painstaking measurements of tau phosphorescence, performed by Longworth, 1961, gave the rates 2.1 ± 0.1. It is characteristical that in the strongly alkaline medium the relative portion of phosphorescence in the total luminescence increases from 50 to 80 % (Vladimirov, Li Chin-go, 1962).

Shortening of the life span of the triplet states and relative (proportional) intensification of the phosphorescence may be associated with

increased probability of singlet-triplet passages (transfers) during ionization of the phenyl group.

Phenylalanine

Phenylalanine, in aqueous solutions, has a long wave band of absorption with the main maximum at 258 mmk (Fig. 27). The absorption band is well structured and "solved" (divided, separated?) in the interval 230 - 280 mmk. into eight elements of vibratory system (structure). However, low rates of molar extinction of the order of 200 indicate partially forbidden character of the electron passage (transfer?), responsible for the long wave band of absorption.

Just as the spectrum of absorption, the fluorescence spectrum of phenylalanine is well structured. In contrast to Teale and Weber, 1957, who failed to solve well the fluorescence spectra, Vladimirov, 1959, Vladimirov and Burnstein, 1960, registered maximums of the vibratory structure at 282, 285 and 289 mmk and the shoulder (arm?) at 303-305 mmk.

The correlation of the individual (separate) elements and their intensity in the fine structure of phenalalanine fluorescence spectrum greatly depends upon the properties of the solvent: in the mixture of acetic acid and ethyl alcohol (1:4) at 77 °K, the principal maximum is 282 mmk, the secondary (collateral) are 273, 288 and 295, whereas in 0.1 H NaOH the spectrum is shifted intop the long wave side and is formed by the main maximum at 292 mmkl. and the maximum at 393 mmk (Vladimirov, Li Chin-g, 1962.

The quantum output of phenalanine fluorescence is low - it is of the order of 0.04, according to Teal and Weber, 1957, and it is constant for the entire stimulation spectrum. In the

strongly Alkaline and strongly acid media there takes place a slight extinction of fluorescence (Vladimirov, Li Chin-go, 1962; Feitelson, 1964)(Fig. 9).

Fig. 27. Phenalalanine, aqueous solution
1 - absorption spectrum (solid line) and stimulation spectrum of fluorescence (dots) (Teal and Weber, 1957); 2 - absorption spectrum (Wetkaufer, 1962); 3 - fluorescence spectrum in the mixture of CH_3COOH and C_2H_5OH (1:4); 4 - phosphorescence spectrum in 0.1 M glycocol at $77^{\circ}K$ (Vladimirov and Li Chin-go, 1962); 5 - luminescence spectrum of the aqueous solution at t= $-140^{\circ}C$ (Burstein, 1964).

Substitution of hydrogen in the benzol ring by halogen (fluorine) is accompanied by a sharp rise in the quantum output - up to 0.4 (Guroff, Michael, Chirigod, 1962).

The life span of phenalyalanine fluorescence was not determined by direct methods, but, judging from the integral band of absorption, it is equal to $8 \cdot 10^{-9}$ sec. (Beaven, 1961).

In the crystalline state, the phenalalANINE fluorescence band is shifted by 15 mmk into the long wave side (Barskii, Brumberg, 1958; Vladimirov, 1959), alsthough in this cases one cannot be sure of the correctness of calculation of errors and spectral sensitivity of the apparature.

Based on the similitude in the spectra of fluorescence and absorption of phenylanalanine, it is conceivable that both bands are formed by one electron passage. The phenylalalanine phosphorescence spectrum consists of three maximums: 424 - 426;

448 - 450; 490 - 495 mmk. (Vladimirov, Burstein, 1960). Although the life span of phsophorescence has not been exactly established, it, apparently, is very small - less than 0.1 sec., from Debye and Edwards data, 1952, and those of Steel and Czent Gyorgyi, 1957. Taking into attention the fact that, according to the findings of Sveshnikov, 1951, both benzol and phenol possess great tau of phosphorescence - 4.1 and 2.2 sec. respectively, - it may be assumed that alanine portion of phenylalanine has the property to remove the quantum-mechanical blocks ("prohibitions") in the trip=let-singlet transfer (passage), as this is done by halogens (chlor-benzol has tau 0.005 , while tribtombrmzol, 0.0007 sec.)

It was shown benzol molecule has not only inhibition ("prohibition") of intercombination, but also for symmetry, and the latter result in the absence of 0-0 transfers (passages) in the fluorescence and phosphorescence spectra (Sveshnikov, 1951).

However, we cannot exclude the possibility that the life span of =hosphorescence of phenylalanine, as quoted by the mentioned authors, is incorrect, inamushas Longworth, 1961, gave the rate 7.3 sec., while Nag-Chaudhuri and Augustine, 1964, that of 5 5 $\pm$ 0 48 sec.

The polarization spectra of phenylalanine have not been studied, nor the orientation of electron passages (transfers).

Chapter II

Electron-Stimulated State of Proteins (Albumines)

Prptein macromolecule contains considerable amount of chromophore groupings, i.e. energetically independent combinations (associations) of atoms, capable of absorbing quanya of light

in the ultraviolet region of the spectrum. By their nature, all the chromophore groupings of proteins may be divided into two large groups: 1) chromophore monomers, i.e. residues of twenty aminoacids which have the property to interact with the light both in free state and in the polymer compound; 2) the chromophores, proper to the polymer itself per se and completely disappearing after dismembering (differentiation or disintegration) of the polymer molecule into its component monomers. In the chromophore of the latter typr belong such atom groupings, as the peptid bond, as well as thyo-ether bond of cystein mercaptids or glutathione of the type

spelling?

$$R_1 - S - \underset{\underset{R_2}{|}}{C=} CH - R_3$$

Table 4 generalizes certain characteristics of the absorption properties of the main chromophore groupings of the protein molecule (these data are those for aqueous solutions).

The acyclic aminoacids have a weak absorption, startgin with 230 mmk.

In the majority of proteins, in the region of 250-300 mmk. the principal portion of the quanta is absorbed by the aromatic aminoacids - tyrosine, tryptophane and phenylalanine. The absorption of aminoacids, containing sulfur, is manifested in the spectral zone 235 -250 mmk. The absorption of the light by the peptid bonds begins to predominate only at the borderline of the passage (transfer) into tje vacuum ultraviolet at 150-220 mmk. If in the ABSORPTION OF LIGHTΔ the leading role is already played by the aromatic aminoacids, then it is even more pronounced in

fluorescence. As mentioned in the introduction, the concept about the luminescence property of all aminoacids without exception proved to be unfit (worthless), as well as that of the property of the protein molecule to luminesce as a whole, similar to the semiconductor crystal (Iordan, 1938; Czent-Gyorgyi, 1941).

Table 4

Chromophore Grouping of Protein

Chromophore	Absorption maximum, mmk	Molar extinction maximum	Long wave borderline in absorption spectrum, mmk	Literature source
Tryptophane (pH 2)	280, 218	6500, 27000	310	Beaven, 1961
Tyrosine (pH 2)	275, 222	1290, 8000	295	Beaven, 1961
Tyrosine (pH 12)	295, 240	2300 10000	330	
Tyrosine (aqueous)	275	1520(1340)	295	Cowgill, 1963 Teale, 1962
Phenylananine	258, 205	200, 8500	270	Beaven, 1961
Histidine	240			Edsall, 1954
Cysteine(alkaline)	235	4000 - 6000	250	Benesch, 1955; Gorin, 1956
Cystine	245 - 249	340 - 360	300	Fromageot, Schenk, 1950
Peptid bond	185	2700, 6500	220	Preiss, Setlow, 1956; Zeidel, 1955; Ham, Platt, 1952
Thio-ether bond	308	7000	-	Brady, 1963
Acyclic aminoacids	-	-	230	Magill et al, 1937
Glycin	-	10 at 220 mmk	230	" "
Leucin	-	50 at 220 mmk	230	" "

In 1957 we showed (Konev, 1957) that the entire ultraviolet luminescence of proteins is conditioned exclusively by the aromatic aminoacids. Studies on a number of plant and animal proteins - serum albumine of man, egg albumine, gamma globuline from pumpkin seed, gamma-globulin of rabbit blood serum, arachine, zein, glyadine, casein, sturnin, calf thymus histone, clupeine, pepsin, bromeline - confirmed that the

spelling?

luminescence property is a a property common to an overwhelming majority of protein bodies which is conditioned by the aromatic aminoacid contained in them, and most of all by tryptophane.

It was found that proteins, not containing aromatic aminoacids, - clupeine and sturnine - do not have any luminescence property either. This immediately discounted from the calculation the possibility of luminescence of the peptid bonds, acyclic aminoacids and other components of protein, as well as possible luminescence, proper to the macromolecule as a whole, in concordance for instance with the proposal made by Perdran and Czent-Gyorgyi, 1941, 1955, About its semi-conductor nature.

The proteins which contain tyrosine and do not contain tryptophane (Nucleo histone, zein), produced tyrosine spectrum of fluorescence. The proteins containing tryptophane - pesin arachnine, egg white and others essentially had a tryptophane fluorescence spectrum.

The second proof of the aromatic nature of protein luminescence in proteins was in the stimulation spectra of their fluorescence in which the tryptophane maximum is pronounced at 280 mmk. Later. this main conclusion about the protein luminescence and the aromatic nature of protein luminesccens was many times confirmed with the aid of various scalar and vector characteristics of luminescence: phosphorescence spectra and spectra of its stimulation (Vladimirov, Litvin, 1960); polarization spectra of fluorescence after absorption (Teale and Weber, 1960; Konev, Katibnikov, 1961); polarization spectra of fluorescence after

emission (Konev, B brovich, Chernitskii, 1964); polarization spectra of phosph rescence of proteins after emission (K nev, Bobrovich, 1964); For c.nvenience of descripti.n, and taking into the account the sharp differences in pr.perties, we follow-ed Weber in dividing (Weber, 1960) all rptoeins int. two large clAsses - class A and class B.. Class A incorporates all those proteins which do not contain tryptophane residues and the spectral luminescence properties of which are c.nditi.ned by tyrosine. Class B c.ntains thr tryptophane containing proteins. Their luminescence is c.nditioned essentially by the residues of this one ~~mmmxmfxthmme~~ aromatic aminoacids

Class A Proteins

(Tyrosine containing proteins)

Protein fluorescence in proteins, not containing residues of tryptophane, but containing phenylalanine and tyrosine, is conditioned exclusively by the residues of tyrosine (Konev, 1957). Proteins of this type have been isolated by Weber into class A (Weber, 1960, 1961)$ Among the studied pr.teins of this class we may mention zein, insulin, tr.pomyosine, ribo-nuclease, trypsin inhibit.r, isomerase 5 3 ketosteroid, ovo-mucoid, nucle.histones, malate-dehydr.genase, .xytosine (poly-peptid). According t. Teale's determinati.n (1960), insulin, zein, ribonucleAse and .vomucoid have tyr.sine maximum fluo-rescence at 304 mmk. In the proteins of ckass A, as well as in di=eptid of phenylalanine-tryrosine, the phenylalanine component is Absent in the fluorescence and stimulati.n spectra (Cowgill, 1963).

Contrary to tryptophane, no conformational reconstructions of the macromolecules are reflected in the position of the fluorescence spectrum of tyrosine. The maximum at 304 mmk. is observed in all of the proteins, both in the native state, and with their denaturation with octo-molar urea. The inclusion of tyrosine into the protein composition is accompanied only by the effect of extinction of luorescence: the quantum output drops down to 8.0; 3.7; 1.75; 1.2 and 0.7 $^{o}/_{o}$ in zein, insulin, ribonuclease, ovomucoid and trypsin inhibitor respectively. It could be assumed that the cause for the reduction of quantum output is in the processes of extinction in the course of tyrosine-tyrosine migration of energy. Actually, the polarization spectra of tyrosine fluorescence after absorption, while they coincide in form with the similar spectrum of tyrosine in free state, still differ from it by their lower rates of the degree of polarization (Wever, 1960). For example, in the case of insulin, polarization is 0.18, whereas in the free tyrosine is it 0.36. The low rates of the f degree of polarization are preserved in proteins also in the propylen glycol aqueous mixture and at the temperature of -70 ^{o}C, i.e. under the conditions which exclude possibility of rotatory depolarization. Theree ore, the effect of depolarization is conditioned by tyrosine-tyrosine migration of energy. Its effectivéity, evaluated by a method described in greater detail for the case of tryptophaneOtryptophane migration of energy, is equal to $\sim 50^{o}/_{o}$. However, still it is not the course of the process of migration that is the cause of the

low rates of quantum outputs of proteins of class A. This is supported by the absence of concentration extiction of tyrosine fluorescence in solid films of polyvinyl alcohol, up to the concentration of 1 m/l, discovered in our laboratory, as well as the closeness of the quantum output of fluorescence dipeptid tyrosine-tyrosine (Cowgill, 1963 B), where the tyrosine residues are in direct contact with the quantum output of otler dipeptids of tyrosine (Table # 5).

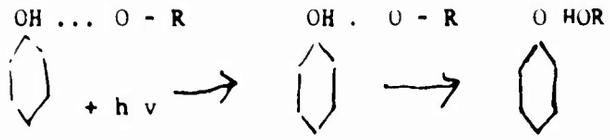
One of the main reasons for the low quantum output of tyrosine residues in protein is the influence of the neighboring electron negative groupings.

Back in 1957 Konev mentioned approximately twice as low quantum output in dipeptid glycyl-tyrosine as compared to the free tyrosine, and he associated the decrease in quantum output with the extinction effect of the nearest peptid bond Later, this point of viewvas substatiated by systematic research by Cowgill, 1964.

Already the presence of three peptid bonds produces decrease in quantum output from 0.21 in pure tyrosine and up to 0.035 in glycyl-tryrosyl-glycynamid (Table 5) The latter rates of the quantum output are quite close to the quantum outputs of protesin of class A.

However, it would incorrect to credit all the effect of extinction of the tyrosine fluorescence of proteins to the influence of the nearest peptid bonds. From this point of view, it would, for instance, be hard to understand why the tyrosine quantum outputs in various proteins strongly

differ among themselves. The second main reason for the extinction of tyrosine residues fluorescence in proteins is the entrance of the phenol group into the hydrogen bond with subsquent ionization of this group in stimulated state and the formation of weakly luminescent phenolate ion:



Usually, the hydrogen bond is formed between OH-group and the ionized carboxyl group by the glutamine and asparagine aminoacids.

A number of factors indidcate the correctness of such a viewpoint. First, tyrosine fluorescence and that of tyrosine-containing proteins is selectively extinguished by substances, containing charged carboxyl groups (Teale, 1961).

Second, the quantum output of tryseein tyrosine fluorescence in copolymer, consisting of 4 °/o tyrosine residues and 96 °/o of glutaminic acid residues, is very low in the neutral solution - 0.02 - and increased with pH=2.0 to 0.3. Therefore, the conversion of the grouping COO^- into the form of COOH, incapable of entering into hydrogen bond with OH-group of tyrosine is accompanued by removal fo the effect of extinction (Rosenheck and Weber, 1961).

Third (in the third place), the degree of extinction of tyrosine fluorescnence in various proteins is correlated with the number of carboxyl groups of aminodicarbon acids (Teale, 1961), proportional to one tyrosine residue (Table 6).

Table 5

Quantum Outputs of Fluorescence of Some Tyrosine-Containing Peptids

HO - CH_2-CH-R_1
|
R_2

Compound	R_1	R_2	Quantum output	Source
Tyrosyl	$-COO^-$	$-\overset{+}{N}H_3$	0.21	1963 (A) Cowgill,
Tyramine	-H	$-\overset{+}{N}H_3$	0.185	"
Leucyl-tyrosine	$-COO^-$	$^{+}H_3N$ - CH - CO - NH - (leuc.)	0.103	"
Tyrosyl-glycyn	$-CO-NH-CH_2-COO^-$	$-\overset{+}{N}H_3$	0.074	"
Glycyl-tyrosine	$-COO^-$	$^{+}H_3N - CH_2 - CO - NH -$	0.070	"
Tyrosine	-COOH	$-\overset{+}{N}H_3$	0.056	"
Glycyl-tyrosyl-glycynamide	$-Co-NH-CH_2-CO-NH_2$	$^{+}H_3N - CH_2-CO - NH -$	0.035	" (B)
Tyrosyl-alanine	-COOH	$^{+}H_3N-CH_2-CO-NH -$	0.09	Cowgill, 1963
ph.? Tyrosyl-f.-alanine			0.08	
tyrosyl-tyrosine			0.08	
Copolymer n (l-glut-l-tyr.) pH 7, 0.2 M NaCl			0.038	Pesce et al 1964
Same, pH 3.0 (alpha-spiral)			0.08	
n(dl-glut-l-tyr_ pH 7 (ball,knot)			0.038	
Same, pH 3 (spiral)			0.069	
n (l-lys-l-tyr) pH 7			0.09	
n (l-lyz-l-tyr-l-lyz) pH 7			0.020	

Table 6

The RAtio of Quantum Output of Fluorescence in Certain Proteins and their Content of Free COO^- groups

Protein	Quantum output of tyrosine fluorescence	COO^- tyrosine residue
Zein	6.0	1.0
Insulin	3.7	1.14
Ribonuclease	1.7	1.4
Human serum albumine . . .	1.0	1.65

In the foutth place, the break (disintegration) of aqueous bonds in proteins, under the effect of mixture of alcphol and hydrochloric acid, results in increase of quantum output of fluorescence (Vladimirov, 1960).

Actually, in the proteins of 5.3 isomerase of ketosteroids, the fluorescence of which is conditioned by four normally titrated resid es of tyrosine (not entering into the hydrogen bond), the quantum output of fluorescence is high and represents 65% Of the quantum output of fluorescence of an equivalent solution of tyrosine (Wang-Shu-Fang et al, 1963).

In this manner, the combined effect of two causes:, the influence of electron negative groupings and, first of all, the peptid, on the one hand, and the specific intereffect with the neighboring charged carboxyl group of aminodicaronic acids, responsible for partial ionization of the hydroxyl group of tyrosine, on the other hand, is what produces the decrease in the quantum output of protein fluorescence in class A. If the nature of the primary, secondary and tertiary structure of various native proteins, as well as chAnges in the secondary and tertiary structures during the

tures during the processes of denaturation has almost no effect on the position of the maximum in the fluorescence spectra of the proteins of class B, then this cannot be claimed with the reference to the quantum output.

With the thermal denaturation, Konev, 1957, observed increase in the quantum output of zein by 14 $^{o}/_{o}$ and Burstein, 1964, decrease in quantum output of insulin by 35 $^{o}/_{o}$.

Thorne and Kaplan, 1963, observed, under the effect of 6.5 molar urea, increase in intensity of tyrosine fluorescence of pig heart malate dehydrogenase.

Cowgill, 1964, observed two-fold increase in quantum output with denaturation of RNK with 8 M or urea. The macromolecular character, conditioned by conformational changes in the macromolecule, of the effect of urea along the diagram of effect: changes in conformation changes in microsurroundings or tyrosine residues changes in quantum output, then, may be confirmed by the absence of similar effect of urea on the tripeptid of glycyl-trysosyl-glycyl-amide, coincidence of the concentration thresholds of the start of the effect of urea on the luminescence and on the process of denaturation (4 M), as well as by the coincidence of the course of dependence of the intensity of fluorescence with the course of dependence of differential spectra of absorption (with lambda = 287 mmk.). The kintics of the denaturation process, evaluated by the intensity of luminescence, corresponds well to the kinetics of the first order.

RNK ? ribonucleic acid?

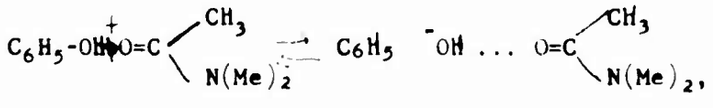
According to the data of Cowgill, 1964, not alone urea, but also other means of disturbing the native structure of the

macromolecule, as well as the effect of certain detergents (dodecyl-sulphate) or the rupture of the bisulphide bridges, which cement the carcas of the macromolecule with the aid of mercaptoethanol, also result in the increase of the quantum output. It is characteristical that also in the latter case, the increase of intensity of luminescence reflects not at all the removal of the extinction effect of the nearest to the tyrosine SH groups, but precisely the structural reconstructions, inasmuch as the break of the neighboring bisuphide bond in polypeptid oxytosine, not having secpndAry and tertiary protein structures, is not accompanied by the increase of quantum output. Gally and Edelman, 1964, from the curves of the ratio of the intensity of fluorescence of ribonuclease and the temperature in the region of structural change (transfer?) found not decrease in fluorescence intensity with rising temperature, but, on the contrary, its increase. The point of structural change at pH 5 was found with temperatures of 57 - 58°C and coincided with the data of optical rotation.

Russian PHK

Painstaking examination of the RNK of the pancreatic gland in larger horned cattle showed that 50°/o of the tyrosine residues possess abnormal properties - they are resistant to the attAck with iodine, have a low constant of dissociation, and do not change differential spectra of absorption with different influences (Scheraga And Ranly - in the bibliography Rupley?, 1962). An analysis of the experimental data, led these authors to the conclusion that this half of the tyrosine residues is screened inside the protein molecule in the hydrophobic cavity. However,

such conclusion at first glance is in contradiction to the low quantum outputs of fluorescence of this ferment, although the presence in the non polar surroudnings, it seems should have the opposite effect. Cowgill, 1964 A, successfully removed this contradiction, showing that in the non polar hexane solutions phenol easy enters into the complex with NN-dimethulacetamide, according to the diagram



and this is accompanied by fluorescence extinction.

Just as the fluorescence, phosphorescence of the proteins of class A is conditioned by tyrosine. Vladimirov and Burstein, 1960, registered in aqueous solutions at the temperature of liquid nitrogen a non structured band of phosphorescence of zein with maximum at 390 mmk. The life span of the stimulated triplet states of the tyrosine in proteins is 2.1 sec (Longworth, 1961).

According to Douzou et al, 1961, histone has phsophorescence maximum 395 mmk abd tau = 1.74 sec.

Konev and Bobrovich, 1964, showed that the heavy atoms (romine and litium iodide) facilitate the triplet-singlet passages of tyrosine in proteins. At the same time, there appears an intensive well structured phosphorescence of the tyrosine residues with maximums of fine structure at 354, 367, 376, and 397 mmk. The elements of the fine structure of phosphorescence reflect the vibratory levels of the basic state.

Class B Proteins

(Tryptophane-containing proteins)

One of the principal features of protein luminescence of this class is as follows. In spite of the fact that in the composition of the proteins there are included three different fluorescent aminoacids - phenylalanine, tyrosine and tryptophane - in the protein fluorescence spectra only the tryptophane maximum alone is manifested (Vladimirov, Burstein, 1960; Teale, 1960). At the same time, in the fluorescence spectra of equivalent amino-acid mixtures of these proteins there is observed luminescence of all three components. Hence, the fluorescence spectrum of protein molecule is not an additive spectrum of the aromatic amino-acids from its composition, but, as a rule, it is formed by only one of them - the tryptopha e fluorescence. This is particularly graphicaliy demonstrated in the study of the ratio of the protein fluorescence spectra and the wave length of the stimulating light. E en back in 1957m we carried out (Konev, 1957, 1958, 1959) a study on this ratio with the aid of the following technical procedure. Fluorescence was stimulated by monochromatic light through spectrophotometer SPh-4, and the spectral composition of the fluorescence was evaluated by comparing the relative intensity of the fluorescence light that passed through the combination of light filters, isolating various spectral areas.

Ø It was found that the spectral composition of protein fluorescence hardly changes with the changed wave length of the stimulating light, i.e. with the change in the proportion of stimulation of phenylalalanine, tyrosine and tryptophane. This

conclusion has been confirmed by taking protein fluorescence spectra with monochromatic stimulation with an equipment, cpn-sisting of two quartz monochromators and cooled with liquid nitrogen photomultiplier (Fig. 28. Consequently, the fluorescence spectra of the majority of tryptophane-containing proteins are conditioned by a single center · tryptophane, - and tyrosine and phenylalanine are found in proteins in a non luminescent state.

The question arises why phenylalanine and tyrosine which do luminesce in free state, lose luminescence property after being included into the polypetid chain of protein? Why the fluorescence of these two aminoacids is extinguished in proteins?

The first natural conjecture on the mechanism of the ex-tinction was that on the course of the processes of migratikon of energy between phenylalanine and tyrosine, on the one hand, and tryptophane, opn hthe other hand (Konev, 1957, 1958, 1959; Vldimivor, 1959; Steel and Szent Gyorgyi, 1958; Vladimirov and Konev, 1959; Vàadimirov and Litvin, 1960). This idea was con-firmed both by the observance of the donor-hostconditions for the resonance migration of energy between these aminoacids, and by the fact that the transfer of energy to tryptophane was re-giestered in the mixed crystals of phenylalanine and tryptophane (Vladimirov, 1957), tyrosine and tryptophane (Vladimirov, 1961).

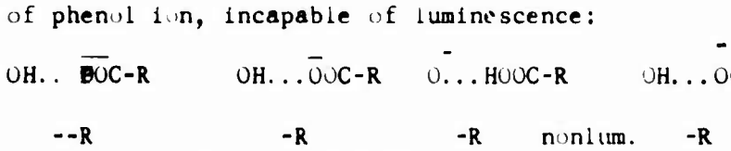
Fig, 28. Fluorescence spectra of the aqueous solution of hemo-trypsinogen with stimulatuon of light of wave length 253, 265, 296 and 302 mmk.

Whether tyrosine actually plays the role of sensibilizator of tryptophane fluorescence in proteins, may be clarified by means of analyzing the spectra of fluorescence stimulation. Vladimirov and Litvin, 1960, Teal, 1960, carefully measured the fluorescene stimulations spectra in a number of proteins, both at the room temperature and at lower temperature (-160-170°C) within the limits of 200-300 mmk. They found that in the stimulation spectra the tyrosine component was absent and the protein stimulation- sluorescence stimulation spectra were determined by tryptophane. In complete agreement with the screening, rather than the sensibilizing role of tyrosine and phenylalanine, the quantum output of protein fluorescence was elevated with wave lengths of 295-310 mmk., where absorption of these two aminoacids is absent (Teal, 1960). Therefore, the stimulation spectra, and the absolute quantum outputs of fluorescence show that the cause of the non fluorescent state of tyrosine and phenylalanine in protein is not the migration of energy. However, one still could believe that tyrosine-tryptophane migration of energy occurs in proteins in such a manner that its quantum output is very low, i.e that the energy of the electron-stimulated state of tyorsine is spent into heat in the very act of energy migration.

Teale, 1960, 1961, produced photo-oxidation of tryptophane of human serum albumine with light of 254 mmk in the presence of methylene blue. The destruction (disintegration) of the only tryptophane residue was not accompanied by the appearance pf fluorescence in 17 tyrosine residues. In another experiment, proteolytic fragmentation of the same protein was accomplished

with hemotrypsin and carboxypeptidase, as a result of which there there appeared protein fragments (debris), containing only tyrosine without tryptophane. However, in this cases again, there was observed no tyrosine fluorescence. Athough a certain percentage of thqu quanta is absorbed (2-10%) by tyrosine, may migrate to the tryptophane - this will be discussed in greater detail in Chapt. III - still hte main reason for the absence of luminescence in ~~tryosine~~ tyrosine in proteins is in the nature of the state of this aminoacid, rather than in the migration of energy.

Just as in the case of proteins of class A, the cuase for the extinction of tyrosine fluorescence in the proteins of class B is in the capacity of the hydroxyl group of the benzol ring to enterinto hydrogen bond essentially with the charged carboxyl groups In the stimulated state, there takes place a break-off of the phenol hydrogen and ionization of tyrosine - formation of phenol ion, incapable of luminescence:



As mentioned above, tyrosine fluorescence is selectively extinguished by compounds which contain chraged carboxyl groups (Teale, 1960). The formation (or appearance) of the tyrosine-carboxyl hydrogen bonds increased the pK of ionization in the phenol group (Lashkovskii, Scheraga, 1954). Measuring of the curves of protein titration shows that the pK of tyrosine ionization in them is actually shifted into the alkaline side, for instance in ribonuclease one half of the tyrosine residues with

pK 10 2 and one half with pK 11.5; in egg white albumine, all phenol groups of tyrosine have pK 11,5.

In favor of the bound state of tyrosine are also the absorption spectra, from the data of Crammer and Neuberger, 1943, the appearance of the maximum of absorption of egg white albumine in the region of 292 mmk with ionization of the phenol groups of tyrosine occurs with pH 12,5 instead of 10.

Two titration eaves - the normal and the abnormal - have been later observed in many proteins, including hemotrypsinogen (Herman, 1963), ribonuclease (Blumenfeld, Levy, 1958; Herman, 1963), myosine (Stracher, 1960, lysozym (Tanford, Wagner, 1954; Donovan, Lashkovskii, Scheraga, 1960)$ Trammer and Shugar, 1959, came to the conclusion that tyrosine in proteins may be found in three different states: tyrosine, free, being in conact with the aqueous phase; tyrosine, bound with hydrogen bonds and dissociating at pH 12 and after thermal denaturation; tyrosine, extremely strongly bound and playing an important role in maintaining the secondary structure of macromolecule.

Finally, the nature of the non fluorescence state of tyrosine is definitively confirmed in the proteins by the exquisite experiments of Vladimirov, 1961, who showed that the break of the hydrogen bonds of tyrosine with carboxyl groups in protein is accompanied by immediate increase in quantum output almost up to the same rates that tyrosine has in its free state.

Protein Fluorescence Spectra

The fluorescence spectra of the various proteins of the class B, therefore, are conditioned by the one and the same

chemical center - tryptophane. And at the same time, sudies on the fluorescence spectra of various individual proteins confirm the idea that each protein possesses a set of tryptophane residues which differ intheir physical and chemical states. This is manifested in the various positions of the maximum of fluoresce ce spectra of various proteins (Table 7). The position of the maximums in the fluorescence spectra vary within quite wide margins between 328 (edestin) and 342 (Oxen serum albumine) mmk.

Table 7 shows the common peculiarity of fluorescence spectra of proteins as compared to the free tryptophane: their shift into the short wave side by 10-20 mmk.

The power of the dipole-dipole and ion-dipole intereffect between the stimulated tryptophane molecule and the molecular surroundings in protein is lesser than in the aquenous solution of aminoacid. On the averAge, the tryptophane residues in rptoein has less polar surroundingsmicro-environment than in the aqueous solution. Precisely, the number, the nature and the character of arrangement in space around tryptophane, ot the polar groups, essentially determine the position of the maximum of fluorescence for the given protein. Since such microevironment may contain a whole number of ionogen groups - alpha, beta, gamma carboxyl, alpha and eta amine, guanide, imidazol, phenol and sulfhydryl, - the position of the fluorescence maximums must undergo a certain dependence upon the pH of the medium. Actually, for hemotrypsine, Burstein, 1960, observed a shift of the fluorescence bAnd from 332 to 339 mmk with the change of pH 20 to pH 8.6. Unfortunately,

the influence of pH on the fluorescence spectra, thus far, has not been studied systematically. The inner connection between luminescence and isoelectrical point of proteins is manifested in the empirical rule, mentioned by Burstein, 1961: the more alkaline the area of the pl of protein, the shorter wave of fluorrescence does it have in the neutral medium.

Table 7

Position of fluorescence spectra maximums of proteins, mmk (after Teale, 1960)

Protein	Water or water-buff. solution	t liquid nitrogen	8 M urea	Propane 1:2 diol	Protein heat denaturated
Lysozym	341		350	343	
Trypsin	332		350	335	
Trypsinogen	332		350	335	
Hemotrypsin	334		350	338	[1]
pH 2,0	3321				339[1]
pH 4,7	3341				343[1]
pH 8,6	3391				345
Hemotrypsinogen	331 [1]	[1]	350	336	[1]
Human serum albumine	339, 338	317[1]	350	340	330
Ox serum albumine	342 [1]	[1]	350	343	
Egg white "	332, 334[1]	320 ,326[1]	340	336	339[1]
Fumarase	335				
Carboxypeptidase in 10% acetate	340		348	340	
Pepsin	342		350	344	
Fibrinogen	337		350	340	
Edestin in ' M NaCl	328		350		
Globin hemoglobin	335		348		
Urease, pH5,0	330[1]				[1] [1]
Actomyosin pH 5,5	330 [1]	338[1]			342 . 350
Human gamma globulin pH 7,3	336[1]2		[2]		341[1]2
XCAsein	335		345		335

[1] - Burstein, 1964
[2] - Konev, Lyskova, Saloshenko, 1963

However, possible effect of the micro-environment on the molecule of tryptophane in stimulated state is not limited to the mechanism of formation of hydrogen bond and dipol-dipol intereffect with the neighboring functional groups. Under

Under certain conditions, the tryptophane spectrum of pretein fluorescence undergoes some radical changes in its form - it changes from the single non strucured band into the rich spectrum rich in structural elements (Bobrovish, Komev, 1064)$ While working with crystals of pig pancrease amylase crystals, these authors, along with the usual spectrum of fluorescence of aqueous solutions of ferments with maximum at 328 mmk observed abnormal spectrum of fluorescence of the ferment crystals at the temperature of liquid nitrogen witn the main maximum at 314 mmk and well expressed maximums at 288, 293, 300/5. 307, 325 and 340 mmk (Fig. 29). This unusual for proteins fluorescnces was accompnated accompanied, though, with a perfectly usual phosphorescence spectrum, characteristical for tryptophane. It is quite understandable that the unusual shape (form) of the spectrum of low temperature attracts attention first of all to the proof that it belongs to tryptophane, and to no other centers of luminescence The tryptophane nature of the low temperature fluorescence spectrum of the crystalline amylase follows from a whole group of factors.

Fig. 29. Fluorescence spectra (1), phosphorescence spectra (2), and spectrum of fluorescence effect (3), of amylase crystals and luminescence spectrum of tryptophane crystals (4) at t=-196 ^{o}C

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1. The stimulation spectrum of this luminescence cmmx coincides with the stimulation spectrum of tryptophane, only differing from it by a slight shift by 3-4 mmk into the short wave side.

2. The tryptophane ring whihh is resposnible for the spectral luminensce(luminescence) prpperties, the indol ring in tryptophane, as mentioned before, is capable of producing, in the non-polar solvents a structured band of fluorescence. For indol, in cyclohexane, it is possible to obtain a low temperature spectrum of fluorescence, close to the amylase fluorescence spectrum.

3. The amylase fluorescence spectrum is similar to its spectrum of phosphorescence, with the excelption of two short wave maximums 288 and 293 mmk. Therefore, in the luorescence spectrum of amylase there is manifested the system of energy levels of the main state of the tryptophane molecule proper.

4. The drying of the crystals isolated from the mother liquor of amylase or their solution in water is accompanied by a gradual change of the unusual fluorescence spectrum into the usual one, without any changes in the phosphorescence spectrum.

5. The form (shape) of the fluorescence spectrum of amylase crystals does not depend upon thelength of stimulation wave within the limits of 255-296 mmk. This directly points out to the fact that the fluorescence band is formed by only one, rather than several centers of luminescence. Other centers of luminescence, beside tryptophane, in proteins (for example, tyrosine, phenylalanine) do not participate in the formation of this unusual spectrum, for in the opposite case there would be dependence of the form of the fluorescence spectrum upon the wave length of the stimulating light.

Thus, all hthe elements of the structure in the amylase

fluorescence spectrum, represented in Fig. 29, belongs to tryptophane.

In this connection, naturally, there arises thequestion as to the causes which result is such a marked modification in the form of the fluorescence spectrum of tryptophane residues. First of all, the fact of the conversion of the unusual spectrum into the usual, with destruction of the crystalline structure of the crystals (with drying in the air, or dissolving in water) indicates that the physical and chemical environment of the tryptophane residues in protein, included in a crystalline network, is different from that of the same macromolecule in solution. At the same time, the intereffect of tryptophane molecules (essentially, the formation of hydrogen beonds between the iminogroups) which takes place during the tryptophane crystal packing proper, cannot be considered as the cause for the changed spectrum. This even follows from that fact that the tryptophane crystals have the spectrum of low temperature luminescence, sharply differing fromthat seen in amylase. The spectrum of low temperature luminescence of tryptophane crystals represents a single non structured band with maximum at 340 mmk. The peculiarity of this spectrum consists also in complete absence of the usual violet phosphorescence of tryptophane. Instead of it, there is observed a weak phosphorescence, only apparent with the phosphoscopical measurements, with maximum at 500 mmk; this does not take p lace in case of amylase which hAs the usual intense blue-violet phosphorescence. Therefore, the state of tryptophane in the crystal net work of protein is different both in the luofrescence spectrum

and the phsophorescence spectrum from that of tryptophane in its own crystalline network.

In this manner, the changes in the spectral properties of tryptophane in the composition of amylase crystals are conditioned by crystallization of the protein molecules proper. Generally, this means that the proteins in the composition of the crystal and in the solution are not qualitatively not equivalent to each other. Whether this may be associated with the changes in the dergee of inner polymer hydration, with close contact and mutual penetration of the polyptid chains of the macromolecules ϕor even with the reversible changes of secondary or tertiary protein structure at the moment of crystallization, this is difficult to say at this moment. At any rate, it may be assumed that tryptophanr luminescence brings with it certain information on the condition of the protein as a macromolecules as a whole and that crystallization causes changes in this state (condition_ - either structural or physico-chemical changes.

Fig. 30. Fluorescence spectrum (1) and polarized spectrum of fluorescence after emission (2) of amylase crystals, t=-196°C. Stimulation 265 mmk.

Conclusion onthe possibility of certain differences of the conformation of protein in solution and in crystal may also be deribed from the data on the tritium hydrogen exchange in solution and in crystal of insulin (Praissman, Rupley, 1964). But it is still not clear what concrete causes can condition such a marked appearance of structure in the tryptophane residues. Apparently, we should look for certain analogy between

the appearance of clear-cut fluorescence structure in amylase crystals and the Shpol'skii effect (1962). To obtain a quasi-linear electron spectrum (Spol'skii effect), it is necessary as we know, to include the activator into the crystalline network, which corresponds in size and geometry to the activator molecule which guarrantees hard (solid) fixation and similar disposition. It may be assumed that a similar situation occurs for the tryptophane residues, surrounded by a mosaic of polyprit and lateral chaines of amylase, being in a crystalline state. Based on this, the fluorescence spectra, presented in Fig. 29, should be considered as non-distorted molecular spectra, not distorted by intereffect, of tryptophane itself. The competence of this conclusion is also seen the resemblance of the amylase fluorescence spectrum to the indol fluorescence spectrum at low temperature in non polar solvents and to phosphorescence spectrum of indol and tryptophane in polar and non polar solvents. The presence of two short wave maximums in the fluorescence spectrum of amylase crystal, absent in the spectrum of its phosphorescence, may be explained, as mentioned above, by the beloning of these two maximums to the electron passage (transfer) ^1L_b in fluorescence. This conclusuon is well supported also by measurements of the polarization spectrum of fluorescence in amylase crystals after emission (Bobrovich, Konev, 1964 ✱ B) which show that both these short wave maximums have much greater degrees of polarization ($10^{0}/o$) than the entire remaining portion of the spectrum ($5^{0}/o$) (Fig. 30).

Thus, the example of amylase in crystal state shows that the real environment of tryptophane (polypeptid chain and its various functional groups) can, by means of constant and induced dipol-dipol intereffects not only decrease the energy of singlet stimulated state and, evening it out, distort the structure of the spectra off fluorescence, bu also it may give the opposite result. The hard congruent fixation of the tryptophane residues in this case guarrantees the appearance of the fluorescence spectrum of tryptophane, close to the molecular.

Quantum Output of Fluorescence and Protein Macrostructure

The quantum output of fluorescence in tryptophane residues of protein is sensitive to their macrostructural organization and, as a rule, possesses a lesser absolute rate than the aqueous solutions of the free tryptophane (B 0.2). Some idea on the rates of quantum output of fluorescence of various proteins is given in Table 8.

The very first surmise as to the causes of the changeability of the quantum output of protein fluorescence was the assumption that processes of concentrationextinction could occur in them, as a result of intertryptophane migaration of energy. This hypothesis was expressed by the author in 1957 for the interpretation of changes in quantum output of protein fluorescence during denaturation and it was supported by VlAdimirov 1957 who found concentration extinction of fluorescence in the aqueous solutionf ot tryptophane.

However, experience of recent years showed that migration of energy between tryptophane molecules is not accompanied by concentration en

concentrational extinction. In the solid film pf polyvinyl alcohol, the concentrational extinction was not observed up to the concentration of 1 M (Konev, Katibnikov, Lyskova, 1963), i.e. up to such concentrations which much exceed the real tryptophane concentration in proteins. At the same time, tryptophane fluorescence in the concentrations of 1 M was almost completely depolarized which indicates the involvement of about 98 % of tryptophane residues in the migration process. Likewise, dipeptid of tryptophane-tryptophane has a quantum output of 0.09, i.e. the same as in dipeptid tryptophane with acyclic aminoacids (Cowgill, 1963, B). Teale, 1961, ascribed particular importance fo the tryptophane fluorescence extinguishing effect of the charged group of amines (NH_3^+), Cowgill associated fluorescence extinction with the influence of the nearest to tryptophane peptid bond as an electron negative grouping.

As mentioned above, partial or complete removal of hydrogen from the iminogroup of indol ring opens up an effective way for the non radiation deactivation of the stimulated states of tryptophane. On the other hand, if we take into account that glycyl tryptophane and acetyl tryptophane-amide, considered As "model" residues of tryptophane in protein, have lesser quantum outputs as compared to it and that proteins may have even higher rates of quantum output than tryptophane itself (casein granules in milk B=0.5, Konev, 1965), then we must admit also the existence of such intereffects within protein which result in increased

quantum output of fluorescence in tryptophane residues.

Table 8

Quantum Output of Fluorescence of Tryptophane-containing Proteins and Peptids (after Teale, 1960, lambda stim. 280 mmk) B_1 -with calculation of protein absorption; B_2 - with calculation of absorption of tryptophane alone

Protein (Peptid)	Quantum Output				
	water		heat denat.	1 8 M urea	
	B_1	B_2		B_1	B_2
Lysozym	6.0	6.5		4.1	4.4
Trypsin	8.1	12.6		13.8	21.4
Trypsinogen	8.7	13.4		14.8	22.0
Hemotrypsin	9.5	10.5		20.4	22.6
Hemotrypsinogen	7.2	8.0	[1]	20.0	22.2
Human serum albumine	7.4	38.0	6.0(pH 12)	5.2	26.2
Oxen serum albumine	5.2	47.5	[1]	7.4	23.0
Egg white albumine	12.1	20.9	8.3	13.1	22.4
Fumarase	9.0	22.1		9.0	16.4
Carboxinogen	12.2	25.0		10.2	20.0
Pepsin	12.8	20.6		14.0	20.6
Fibrinogen	18.0	23.0		12.0	23.5
Edestin (1M NaCl)	11.8	14.3		8.0	11.4
Globin	10.0 [2]				
Glycyl tryptophane	0.05 [2]				
Alanyl tryptophane	0.06 [2]				
Leucyl tryptophane	0.08 [2]				
pr rpolyl tryptophane	0.05 [2]				
Ph.-Al. tryptophane	0,06 [2]				
tryptophyl-tryptophane	0,09 [2]				
Tryptophyl-tyrosine	0.12 [2]				
Tryptophyl-glycyn	0.14 [2]				

1 Vladimirov, Burstein, 1960
2 Cowgill, 1963 B

It would appear interesting to associate the amount of the quantum output of fluorescence with the presence in proteins, in various proportions quantities, of two forms of tryptophane residues - the tryptophane inside of the protein globule in hydrophobic microenvironment and the tryptophane on the surface of the globule in hydrophilic micro-surroudnings. Judging from the behavior of glycyl-tryptophane and acetyl-tryptophane-amide, the molecules in the little polar, hydrophobic environment should have higher rates of quantum output than the molecules in the polar,

aqueous surroundings. The existence of these two forms of tryptophane is confirmed by the data in the studies of protein fluorescence extinction with the lithium bromide (Konev, 1964) and potassium iodide (Burstein, 1965), as well as the data of differential spectrometry with gradual oxidation of tryptophane residues in proteins by hydrogen peroxide (Hoshijima et al, 1964). Steiner, Edelhoch and Frattani, 1964, showed that admixtures which diminish the dielectric constant of water (propylene-blycol, sugar, dioxane, dimethylsulfoxide) intensify fluorescence of acethyl-tryptophane-amide and, on the contrary, admixture which increase dielectric constant (glycyl, glycyl-glycyl) weaken the fluorescence. At the same time, the initial slanting of the curves ~~dependxxpn~~ of dependence of intensity of fluorescence upon the concentration of propylen-glycol in acethyl-tryptophane is much greater than in proteins; this indicates inaccessibility of the tryptophane residues, situated inside of the protein molecule, for the solvent. However, it is suffieient to loosen up the protein structure by restoring the serum albumine of oxen in 8 M urea or with tryptic partial proteolysis of tyreoglobuline in 7 M urea, in order to equalize the slants of the curves of protein and acethyl-tryptophane-amide.

Although the existence of these two forms of tryptophane in proteins is incontestable, we believe it rather risky to insist on a higher fluorescence output in the "hydrophobic" form as compared to the hydrophilic. As a matter of fact, judging from the marked shift into the short wave side of the fluorescence band of trypsin, trypsinogen, hemotrypsin and hemotrypsynogen

contain the greatest comparative quantity of "hydroph bic" tryptophane. However, the quantum utput of fluorescence of in these proteins is not at all high - of the order of 10%, and rises to 22%, only after the effect of 8M urea, i.e. after conversion of the tryptophane residues essentially into the hydrophilic state The great effectivity of the non radiation extinction, in these proteins, is also confirmed by the low rates of tau of fluorescence (Table 9). Hence, there is a contradiction: on the one hand, the little polarized solvents increase the intensity of fluorescence, both acethyl-tryptophane-amide, and the tryptophane residues proper in protein, and on the other hand, tryptophane residues in hydrophobic regions may have lower rates of outputs than in the hydrophilic regions.

This contradiction may be eliminated, if we assume that in the hydrophobic regions, in spite of the effect of low dielectric constant of the medium which increases fluorescence there arise some specific intereffect with the neighboring troups accompanied by processes of extinction. As a result, the "hydrophobic" tryptopane has, as a rule, a low output. Apparently, under real conditions, the quantum utput of protein fluorescence reflect low average rates of outputs of all trypt phane residues, situated at different physico-chemical states, and it reflects the result of several constant or induced dipol-dipol or ion-dipol effects from the surrounding groups on tryptophane in the lower singlet electron-stimulated state. All this, results in a wide diapason of changes in the quantum output of tryptophane fluorescence in protein: from 0.05 in wool keratin, to 0.5 in casein granules of milk. Considering this, it may be

assumed that there must be a close relationship between the intensity of protein fluorescence and their secondary and tertiary structure, essentially predetermining the specificity and the intensity in the intereffect oftryptophane residues, and the microenvironment. Below, we shall review in greater detail the essential complex of data which demonstrate such a relationship and then to describe the entire volume of information supplied by luminescence on the structural organization of the protein macromolecule.

Table 9

tau, quantum outputs (B) and position of maximum ($\lambda_{max.}$) in Fluorescence of certain proteins

Protein	In water				in 8 M urea			
	tau $\cdot 10^{-9}$ sec	B, %	λ_{max}	ta/B · 10	tau $\cdot 10^{-9}$ sec.	B · %	$\lambda_{max.}$	tau/B · 10
Pepsin	4.5	25	342	1.80	3.6	20	350	1.80
Pepsin pH 9/0					4.0			
Hemotrypsin	3.0	10.5	334	2.85	3.7	22.6	350	1.64
Same, after t-denaturation	2.5				3.3			
Hemotrypsinogen	1.6	8.0	331	1.98	3.4	22.2	359	1.53
Globin hemoglobin	3.0	14.3	335	2.08	3.3	11.4	348	2.85
Same after t#denaturation	2.7							
Trypsin	2.4	12.0	332	2.00	3.5	21.4	350	1.63
Human serum albumine	4.1	22	339	1.90	-	26	350	-
Casein	3.7	-	-	-	-	-	-	-
Cytochromine C	3.5	-	-	-	-	-	-	-
Edestin in 1 M NaCl	3.0	23	328	1.30	-	23	350	-
Tryptophane	3.0	20	348	1.50	3.5	-	-	-

Conformation (Configuration) and Luminescence of Protein

The earliest data on the effect of the secondary and tertiary structure in macromolecule on the ultraviolet fluorescence of protein were obtained by us in 1957.

During the study of the intensity of fluorescence of native proteins and proteins with destroyed secondary and tertiary structure, due to thermal denaturation, it was noted that there is increase in intensity of fluorescence in case of gamma-globuline/rabbit/serum, with stimulation by wave lengths 254-290 mmk. Later (Konev, 1958 A and B) increase in quantum output of fluorescence was found for arachine and egg white albumine, but not found for pepsin, gliadin, zein and casein. On the contrary, in case of casein, beginning with the rates of pH 9-10, there was observed decrease in quantum output as result of alkaline denaturation (as will become apparent later, in this case, besides denaturatuon, there were other effects in force, in particular, changes at the level of the quarte- quayernary structures). The changes in quantum output of fluorescence during thermal denaturation were confirmed in thorough experiments by Vladimirov and Burstein, 1960. The influence of conformational reconstructions under the effect of high temperatures and changes in pH were studied by Steiner and Edelhoch, 1961.

The most interesting thing in these authors" observations may be considered to be the fact that they succeeded in showing a good correlation between the region of changes in luminescence intensity and the region of conformational changes (conversions).

The curves of the ratio of fluorescence intensity and temperature (Fig. 31) are nearly linear for the region where there are no structural reconstructions; at the points of structural changes the slant changes, i.e. the intensity of fluorescence

changes in the course of time without any further rise in temperature. After passing the zone of this change, there appeared hysteresis, i.e. with reverse lowering of temperature the whole curve was shifted.

In the gamma-globuline of rat blood serum at pH 11.2 in 0.1 M potassium chloride, starting with 35°C, the slant of the curve $I^{fl} = f(t, c^0)$ becomes more and more negative. With coolong down after reaching the point 60°C the reverse branch is situated above the straight line. For neutral solutions of the same antibody which, judging by the biological tests, has no structural reconstructions up to the temperature of 60°C, the straight branch completely coincided with the reverse branch. The same thing was observed also in lysozyme in which in temperature interval 15-55°C and with pH 2.3 - 6.4 there are no changes in the structure (Tanford, Wagner, 1954; Yang and Foster, 1955; Donovan, Lashkovskii, Scheraga, 1960).

Fig. 31. Depemndence of fluorescence intensity upon the temperature of protein.

a- Bence-Jones albumin, phosphate buffer pH 7 and various concentrations of urea (Gally and Edelman, 1964; b - hemotrypsinogen in water (1) and at pH = 1.9 (2) (Steiner and Edelhoch, 1963).

Judging from tests, independent from luminescence, hemotrypsynogen at pH 2 undergoes irreversible thermal denaturation already at the temperature of 35°C. Accordingly, aith pH 1.9, there is observed a marked anomaly in temperature extinction - retardation of the speed of extinction, caused by structural reconstruction (Fig. 31, a). Edelman and Gally, 1962, 1964,

discovered abnormal curve of temperature extinction for the Bence-Jones albumins, associated with the structural transformation. In the area of the structural transformation with the threshold temperature at 53°C there is observed not the extinction, but growth of intensity of fluorescence and s shift of the maximum of fluorescence from 330 to 340 mmk. With the reverse cooling of the proteins there was observed marked phenomenon of hysteresis (Fig. 31, 6).

With heating of alcohol dehydrogenase of yeast, starting with 36°C, there takes place a shift of the maximum of fluorescence into the long wave side and drop in intensity of fluorescence. Changes in the parameters of luminescence are correlated with the decrease in fermentative activity (Brandt, Evers, Kaplan, 1962).

In alcohol dehydrogenase of the heart the temperature s threshold of changes in fluorescence intensity (60°C) coincides with the thermal thresholds of changes in optical rotation and abatement of ferment activity.

Gerstein, Van Vunakis and Levine, 1963, compared one of the most sensitive tests for the tertial structure of protein - the the immunological antigen - antibody reaction - with the luminescence test. In their experiments on rats, they prepared antiserum for pig pansinogen. They found that with heating of pepsinogen, its antigen activity weakens side by side with decreased intensity of fluorescence.

Perlmann, 1964, observed temperature denaturation of pepsinogen which prevented its transformation into pepsin. The temperatures of denaturation trn

temperatures of denturation transformation, determined by the increase in luminescence intensity, on the one hand, and optical rotation and capacity to activation into pepsin, on the other hand, were found to be very close. Addition of urea in the concentrations: 0.5; 1.0; 1.5; 2.0; 2.5; 3.0; amd 3.5 M brought about gradual shift of the transfer temperature into the side of lower temperatures, identical in all of the tests. More detailed research showed greater sensitivity of luminescence as compared with to the optical rotation to the biologically significant con# formational reconstruction: after 15 min. of heating to 60°C and subsequent rapid cooling down to 25°C, the optical activity was restored only by 60°/0, whereas the fluorescence and the biological activity were fully restored. The intensity of luminescence, after cooling, is restored to the initial level not immediately, but gradually with constant speed $23.1 \cdot 10^{-2}$ $^{-1}$ min. The same is observed also for the biological activity. Starting with 50°, with pH 7, in the trypsin inhibitor, there occurs a structural transfer into the strongly open state with gradually increasing in time optical rotation and viscosity, decreased time of relaxation, increase in negative maximum of differential spectra of absorption at 291 and 286 mmk and increase fluorescence intensity. The structural transformation is governed by simple kinetics of the first order. The kinetical curves of luminescence intensity coicide with those of viscosity and differential spectra (Steiner, Edelhoch, 1963).

Side by side with the temperature conformational reconstructions, the luminescence intensity also reflects structural

chANGES IN THE MACROMOLECULEΔ CAUSED BY HIGH concentrations of urea and changes in pH. Teale studies the quantum outputs of fluorescence of many proteins in native state and after denAturation with 6 M urea (Table 8).

For the majority of proteins, trypsin, trypsinogen, hemotrypsinogen, egg white albumin, fibrinogen, edestin, with addition of 8 M urea, there occurs an increase in quantum output of fluorescence, but it is not equal for different proteins. For example, in edestin, the quantum output only increase by $2^{o}/o$, whereas in hemotrypsinogen by $180^{o}/o$. An insignificant portion of the studied proteins showed decrease in quantum output (lysozym, carboxypeptidase, pepsin), while fibrinogen did not change in this respect during denaturation with urea.

The properties of the tryptophane microenvironment, not identical in various individual proteins (albumins), become levelled down after denaturationwith urea, and the quantum output acquires the rates of 0.2 - 0.26, characteristical for tryptophane in free state. The maximum of fluorescence in the majority of proteins also becomes stabilized at the level of 348-350 mμk. This permits us to use the amount of change inquantum output of fluorescence during denaturation as one of supplementary means for identification of individual albumins (pr~etins).

Velik, 1961, noted quite high rates of quantum output of fluorescence in dehydrogenase of lactic acid (apparently, of the order of 0.7) and its drop almost 4 times after the effect of 6 M urea. Gally and Edelman, 1964, observed structura transition in Bence-Jones albumins, accompanied by increased intensity f fluorescence, under the effect of 8 M urea at tem-

peratures below room temperature.

In 9 M urea, with pH below 4.5 and above 10.5, at room temperature, and with pH 7 at temperatures above 40°C, lysozym undergoes, in time, some structural reconstruction. The kinetic curves of luminescence transition coincide with the curves of optical rotation and differential spectra. The effect of the influence of the structure upon the intensity of fluorescence is quite great: with pH 12.25 in 9 M urea and 0.01 M KCl at 25°C during 8- 10 min. the intensity of fluorescence increases 2.5 times (Edelhoch and Steiner, 1962; Steinter, Edelhoch and Frattali, 1964).

Pepsinogen undergoes structural transsition ar room temperatures in 4 - 9 M urea solutions and pH 6-7; this is accompanied by increased optical rotation, developing in time, increase in negative peaks in the differentiation spectra of absorption at 287 and 293 mmk., weakening of fluorescence (by 40°/o). The constant of speed of this reaction of the first order, calculated from luminescence measurements, fully corresponds to the constant, calculated from the data of differentiation spectra and the optic al rotation (Steiner, Edelhoch, Frattali, 1964).

The parallelism and the decrease in intensivity of fluorescence and in ferment activity is observed under the effect of continuously inceeasing concentrations of urea within the limits of 0 - 8 M for the lactic acid dehydrogenase of the skeletal muscle and heart in ox and chick (Brand, Evers, Kaplan, 1962).

The constants of speed of ther structural transformation, caused by

caused by urea, in lysozym, pepsinogen and tripsin inhibitor of soybean proved to be the same, judging from the measurements of fluorescence, on the one hand, and measurements of the optical rotation and differentiation spectra, on the other hand (Edelhoch, Steiner, 1964).

Another problem studied was that of the effect of the third basic method of denaturation on the quantum output of fluorescence, - denaturation produced by the changes in the reaction of the medium (Konev, 1957, 1959; Steiner and Edelhoch, 1963(.

Especially significant results in this respects were those obtained for lysozym. In aqueous solutions, this ferment does not change its xonformation within the diapason of pH 2 -12 and accordingly it has coinciding curves of the direct and reverse titration - without hysteresis. At the same time, within the interval of pH 6.0 - 8.5, the quantum output is constant, slightly inclined to the acid side and hhen somewhat more so into the alkaline side, starting with pH 8.5 (Steiner and Edelhoch, 1963).

The matter is quite different, if the ferment is titrated in 9 M urea in the presence of 0.01 M potassium chloride. Under these conditions, in the alkaline medium, there is observed a sharp increase in the intensity of fluorescence, spontaneously developing in time. With pH 12.25 during 8-10 min. there is seen increase in fluorescence intensity 2.5 times, with pH 2.75 fluorescence intensity increased by 80^{o}/o. At the same time, the most interesting thing in Edelhoch and Steiner's experiments is the fact that (1962) they succeeded in demonstrating the

complete parallelism in the changes of fluorescence intensity, optical rotation and changes in the point of 293 mmk in differential spectra of absorption which reflect that dependence of these indices upon the pH with direct and reverse reversible titration. Therefore, these experiments showed that the changes in fluorescence intensity directly reflect the structural reconstructions in the macromolecule.

A considerable effect of extinction of fluorescence and the phenomenon of hysteresis between the curves of the direct and reverse titration were found in egg white albumin, starting with pH 12.6, in oxen serum gamma-globuline, starting with pH 11.5, in pepsin, starting with pH 7.5 - 8.5, As a rule, the points of structural transformation, identified in luminescence as the threshold rates of pH with which begins the hysteresis of direct and reverse titration, corresponded well to the points of structural transformation, demonstrated with the aid of other methods: with solution (solubility), optical rotation, stain dye sorption, or ferment activity.

In this manner, two main luminescence tests - temperature (or concentration) threshold, beginning with which there are observed changes in luminescence intensity, for the most part irreversible (phenomenon of hysteresis) and kinetic curves of these changes are in good correlation with other tests for the structural reconstruction of proteins (test of optical rotation, differential spectrophotometer, viscosity and, what is particularly important, with the direct tests for biological activity). At

the same time, in a number of cases, the luminescence intensity proves to be a more sensitibe test for structural transformation than other methods commonly used for that. We may cite three examples.$ The soybean inhibitorof trypsin (Edelhoch and Steiner, 1964 detects with acid pH only with difficulty registered changes in optical rotation, differential spectra and polarization, whereas the luminescence intensity chAnges with gr .ter contrast - its increase may be up to 30°/o/

In the oxen serum albumine in the interval of pH 7-9 no changes are detected in the optical totation, although changes are observed in the capacity to bind calcium and methyl orange stain ions. This lead to the idea on the existence of a structural transformation under these conditions. Actually, Steiner and Edelhoch, 1963, with mentioned pH deunf found considerable extinction of fluorescence of oxen serum albumine.

With repeated heating and cooling of pensinogen, the optical activity each time if completely restored, although biological activity gradually abstes. After five cycles of heating and cooling, the biological activity is decreased down to 70°/o of the initial rate, and in compiete agreement with this, the fluorescence intensity becomes equal to 64°/o. Therefore, in the given case, the luminescence test proved to be more adequately related to the biological test than the test of the optical rotation (Perlamnn, 1964).

In other cases, luminescence if less sensitive to the conformational transitions than the optical rotation, differential spectrophotometry or viscosity.

At the same time, luminescence has one incontestable advantage as compared to other methods of structural analysis # - that is the possibility of working with infinitesimal amounts of albumin. Moreover, the presence of other substances is no little hindrance to luminescence measurements; this, in future, may serve as basis for measuring conformational reconstructions directly in the live, functional cell.

All this permits us to consider the phenomena of albumin luminescence as a very useful auxiliary method for the study of conformational reconstructions in the protein systems.

During recent years, the point of view on the dynamic nature if the secondary and tertiary structures of the protein in ferment at the time of its functioning is more and more prevalent among the enzymologists.

It is believed that the marvelously accurate geometrical correspondence of the active center of the ferment and substratum does not precede preliminarily, but occurs in the course of the intereffect of the ferment and the substratum (the hypothesis of induced contact, of Koshland, 1962).

It is assumed that (for instance, Eigen, Gordon, Hammes, 1963) the conformational changes in the mAcromolecule are indispensAble for the selection of the optimal (critical) distribution in space, for substratum and co-ferment, with which the bond intended for breaking is most effectively polarized and broken. It is quite probable even that the conformational changes per se may cause weakening and break (rupture) of the bond (as though "loaning" of energy at the moment of over-

spelling?

coming of activation barier).

As reported by these authors, with the aid of relazational methods, it is possible to record several types of conformational chAnges in the serum albumine during the time of the order of 10^{-3} sec. Eigen, 1964, et al showed that the period of time of spiral - ball, for the polyglutaminic polyglutamine acid and polylysine is less than 10^{-7} sec. Such a high speed of conformational changes makes it quite possible that they play a role in catalysis It may be considered particularly worthy of attention that as a matter of fact one of the earliest data on potential changes in the microenvironment yptophane residues in the protein mAcromolecule at the moment of "work: were obtained precisely wuth the luminescence method white udying the luminescence spectra of ferment-substratum complex of urease - urea; at that time, Bursteinm 1961, discovered for that complex a somewhat mixed spectrum for this complex, as compared to non working ferment spectrum. In other words, the macromolecular micro-environment for the tryptophane residues in urease at the moment of fermentolysis was found to be changed, i.e. one could assure changes in the conformation of the entire macromolecule (or the active center). Soon afterwards, there were obtained some results, demonstrating changes in the quantum output of fluo-rescence of alpha-hemotrypsin, after addition (to it) of a number of synthetic substrata and Mi inhibitors (Stutervant, 1962).

Later, chAnges in the conformation of the ferment macro-molecule, with the formation of the ferment-substratum complex,

were detected with direct methods - methods of dispersion of optical rotation for hemotrypsine (Havsteen, Hess, 1962), for transamynase of glutaminic acid (Fazalla, Gammes, 1964); for oxydase of d-aminoacids in the complex with the co-enzyme - flavin-adenindinucleotyd - and in the triple complex with coenzyme and artificial substratum - benzoate (Yagi, Ozawa, 1962) and others.

In this manner, the intensity of luminescence reflects not alone the denaturation, considerably artificial conformational Alterations in the protein macormolecule, but also those natural changes of the secondary and tertiary structure which occur in the working mechanis, propse proper during the functioning of the ferments.

Judgins from the data of luminescence, similar ratios are also obser ed in the antigen-antibody reaction.

The most interestin data, in this connection, were obtained with botulism toxin (Borov, Fitzgerald, 1958; Borov, 1959). The botulism toxin (clostridium botulinum A, C, D) represents an albumin with high molecular weight about 900000, easily disintegrating into fragments with molecular weight 45000 - 70000, i.e. actually into separate individual albumin molecules. Included in the composition of botulism toxin is 1.69°/o of tryptophane. The conversion of toxin into 6 M solution of urea resulted in weakening of the luminescence intensity approximately by 40°/o and complete loss of its biological activity. It is probable that changes in the quantum output are conditioned precisely by the disintegration of the large botulism molecule

int› fragments (influence of the quaternary structures).

In the c›mplex of the tw› pr›teins, antitoxin - toxin, the quantum ›utput ›f the latter is much l›wer than in the free state. This is n›t ›bserved in c›mbinati›n ›f serum fr›m n›n immunized animal with t›xin ›r with additi›n ›f antitoxin A to toxin C ›r vice versa.

The last conclusi›n ›f these auth›rs ›n the identity ›f the active center ›f t›xicity and luminescence was severely criticized by Schantz et al, 1960. This author established that in a number ›f cases it is possible to eliminate completely the t›xicity ›f the specimen (substance?) without any remarkable changes in its luminescence capacity (in urea and in guanidine); they als› f›und absence ›f any c›rrelati›n between the intensity ›f luminescence and the biol›gical activity of chr›mat›grphic fractions ›f toxin in cellulose. Hpwever, this criticism considerably misses the target, if we assume that not all tryptophane residues enter int› the active ce ter and that in a similar manner in all ferments, the biological activity is preserved in the toxin only with the presence ›f an active center and the native state of the entire mAcrom›lecule ›f pr›tein as a wh›le.

And, then, finally, the third basic kind ›f functi›ning ›f the pr›tein molecules is the mechs-›-chemical pr›cesses which convert the chemical energy of the mAcroenergetical bonds ATF into mechanical w›rk. The earliest luminescence research on the macr›molecule ›f protein at ›the m›ment ›f functi›ning ›f this type was done by Burstein and Syslova, 1964, and Shtrankfel'd, 1964.

ATF ?

"flowing-through" (meaning: constant flow)

With the aid of a running-through cuvette, Burstein, 1964, Burstein and Suslova, 1964, studied changes in luminescence during the formation and disintegration of ferment-substratum complex of myosin-ATF, according to the diagram:

$$E + S \rightleftarrows ES \rightarrow E + P.$$

The formation of the complex corresponded to the decrease in fluorescence intensity of tryptophane in it, and at the same time the kinetics of changes in fluorescence intensity had two distinct phases - a drop in intensity of fluorescence during the starting period, representing the formation of the complex, and its gradual flare-up back to initial level, corresponding to disintegration. The difference in the stimulation spectrum of myosine fluorescence in the complex and in the free state $\Delta F_{max.} = f(\lambda)$ had its maximums at 274, 283, 5, 291 mmk, appearing against the background of monotonous decrease in the fluorescence complex. These maximums correspond well to= the maximums of differen spectrum of absorption of trypt phane at 274, 283,5 and 291 mmk. It is characteristic that the form of the kinetic curves of protein luminescene changes in its details depending upon the length of the wave of stimulation; this, possibly, reflects some still unmxplained stages of ferment reaction which contribute their share into the general picture, depending upon the spectrum.

In this manner, the secondary and tertiary structure of protein macromolecule has a marked influence, both on the amount of the quantum output of the tryptophane residues included in its composition, and upon their spectrum of stimulation. This

latter circumstance permits to substitute the differential spectra of absorption during the denaturation, conformational reconstructions of protein by the differential spectra of stimulation of fluorescence, fit for work practically with the successive concentrations of substances.

Apparently, not only the secondary and tertiary structure have effect on the intensity of luminescence, but, likewise the higher levels of the structural organization. Konev, Lyskova, Saloshenko, 1963, Konev, 1964, showed that the formation of the quaternary structures - granules in the protein milk casein - is accompanied by increase in fluorescence intensity approximately twice. The disintegration of these granules, caused by various chemical (urea, ammonia sulfate, dodecylk-sulfate) and physical (strong dilution, ultrasound) factors is accompanied by weakening of intensity of luminescence.

Duration of The Stimulated State (tau) of Protein Fluorescence

the tau of fluorescence of a number of proteins are given in Table 9 (Konev, Pikulik, Kostko, Chernitskii, 1965). From this Table it may be seen that of the eight studied proteins only three have identical tau of fluorescence - globin, edestin and hemotrypsin. Therefore, the tau of fluorescence may be considered as a certain individual characteristic of the macromolecule. Study of the tau of fluorescence once again confirms the influence of the macromolecular structure on the condition of tryptophane in protein. Denaturation of proteins with 8 M urea causes levelling out of the tau of proteion fluo-

rescence just as this also occurs in the case of the quantum outputs. At the same time, the tau of fluorescence of proteins because equal to the tau of tryptophane fluorescence in urea - $3,5 \cdot 10^{-9}$. The tau of the protein fluorescence was found to be indppendent from the wave length of the stimulating light from 270 to 300 mmk; this may be considered as a proof of redistribution of energy between the levels ^1L_b and ^1L_a during a period of time, lesser than 10^{-10} sec.

Polarization Spectra of Protein Fluorescence after Absorption

The polarization fluorescence of proteins, measured by Weber, 1960, and somewhat later but independently from him by Konev and Katibnikov, 1961, was found to be considerably lower than that of free tryptophane (Table 15).

The considerably lower rates of degree of polarization, as compared to free tryptophan, were observed not only in the aqueous solutions of proteins, but also in 50% binary mixture of propylglycol-water at the temperature of -70 °C (Weber, 1960), in proteins in solid state at room temperature and at the temperature of liquid nitrogen (Konev, Katibnikov, 1961), in proteins in glycerine and solid (hard) films of polyvinyl alchhol (Konev, Katibnikov, Lyskova, 1964' in casein ~~film~~ embedded into composition of hard granule f milk and in casein film (Konev, Lyskova, Saloshenko, 1963). All this indicates migrational nature of depolarization of protein fluorescence.

The polarization spectra of protein fluorescence after absorption were studied

absorption were studied by Weber, 1960, 1961, 1963; Konev and Katibnikov, 1961; Konev, Katibnikov, Lyskova, 1962, 1964; Konev, Bobrovich, Chernitskii, 1964, Bobrovich, Konev, 1964. Generally speaking, the forms of the polarization spectra of protein fluorescence correspond to each other, with the exception of decreased absolute values for the degree of polarization in all proteins as compared with the free tryptophane (Fig. 32), as mentioned above.

Fig. 32. Polarization spectra of fluorescence after absorption of tryptophane in polyvinyl alcohol (1), human serum albumine (2), and hemotrypsinogen (3) in water, t=20°C, registration 345 mmk

The minimum of polarization spectra in the region of 290 mmk indicates that the tryptophane residues in the protein composition absorb light with participation pf the same two electron passages (transfers) $^1L_a \leftarrow A$ and $^1L_b \rightarrow A$, as the tryptophane in the free state. In some proteins, the tryptophane residues possess a certain predominant orientation of their axes along one of the axes of the macromolecule. In this case, the protein may be oriented, and along with it it is possible to orient the tryptophane residues too. As a result, conditions are created for the polarization "registration" of absorption of the negative oscillator in the manner, as described in the section dedicated to the polarization spectra of tryptophane. Actually, for the wool keratine, and for silk fibrion it was shown that the

negative oscillator

netAtive oscillator ^1L_b forms absorption spectrum with the maximum ar 290 mmk and generally similar to that of free tryptophane (Fig. 33). (Konev, Katibnikov). In a number of prote ns - human serum albumine and oxen serum albumine, pepsin, chemotrypsynogen, dehydrogensae of lactic acid or mouse muscle and lysozym, there is found vibrating structure of the passage (Migration?) ^1L_b, as this occurs in N-glycyl-tryptophane in the propylglycol at -70°C and in indopl in sugar lollipop candy at room temperature. This is expressed in the second maximum of the spectrum at 285 mmk abd minimum atr 295 mmk (Weber, 1960 B)

The amount of this accessory maximum, reflecting the mirco-surroundings of the tryptophane residues, varies straongly with transfer from one protein to another and may serve a very specific qualitative characteristic of various proteins. For example, hemotrypsin has no accessory maximum and minimum, and its pre- cyrsors - hemotrypsynogen which does not differ from it in its aminoacid content, has a marked fine structure of polarization spectrum (Weber, 1960, 1961, 1963).

Velik, 1958, 1961, observed high degrees of polarization of fluorescence (P=0.22 ÷ 0 25) of two ferments - lactic Acid dehydrogenase and glutaminic acid dehydrogenase in the liver, which coincide with the degree of polarization of free tryptophane in viscous media - 0.25 in all cases. Taking into the account the sharp discrepancy of Velik's data and those of other authors, we are inclined to think that some methodical error must have occurred in this measurements results.

Fig. 33. 1 and 3 - polarization spectra of protein fluorescence after absorption for chaotically oriented keratine fibers (wool keratine) with light stimulation, with variations of the electrical vector E in vertical and horizontal xtm directions respectfully, 2 and 4 - the same as 1 and 3, but for the vertically oriented wool keratine fibers; 7 and 8 - the same as 1 and 3, but for the vertically oriented silk fibrion fibers.

Our suspicion is still further strengthened by the fact that judging from the findings of Velik, both ferments have unnatural polarization spectra of fluorescence after absorption in the region of 270-300 mmk in theform of straight lines (Veli,, 1958).

In this manner, an analysis of polarization spectra of fluorescence of prote n after absorption leads to the following conclurions:

1. The long wave band of absorption of tryptophane residues in protein, as well as in the free tryptophane, is formed by two electron passages ^1L_a and ^1L_b, oriented to each other at an angle.

2. The depolarized fluorescence of the tryptophane residues in albumine (protein), as compared to the free tryptophane, shows the course of energy migration.

3. The form of polarization spectra is more individual than the scalar characteristics of proteins, and it is sensitive to the conformational peculiarities in the organization of the macromolecules and the conformational (configurational?) changes, for instance during the denaturation of proteins with urea.

Polarization Spectra of Protein Fluorescence after Emission

Polarization spectra of protein fluorescence after emission have been obtained in 1964 by Konev, Bobrovich and Chernitskii, owing to the use of photomultiplier, cooled down to the temperature of liquid nitrogen and working in the regime of the photocurrent meter (Vladimirov, Litvin, 1960). The polarization spectra of a number of proteins are represented in Fig. 38 and 18, b. The polarization spectra of protein fluorescence after emission may be characterized by two principal peculiarities: first, constancy of the degree of polarization (with a slight drop into the long wave side) for the entire main portion of the fluorescence band, including its maximum; second, increase in the degree of polarization in the short wave portion of fluorescence band within the limits of 310 - 295 mmk. Such a form of the spectrum shows the complex nature of the center of luminosity and it means that the short wave portion of the band is conditioned by other electron passage (transfer) than all remaining portion.

The fact that the short wave luminence, with the shorter span of life, and therefore having lass depolarization, actually belongs to tryptophane, rather than some other centers, may be proved by two factors: coincidence of the center of stimulation of fluorescence at 300 mmk with the spectrum of stimulation of fluorescence at 340 mmk, on the one hand, and independence of the entire band of fluorescence of proteins from the wave length os the stimulating light, on the other hand.

Consequently, proteins, as well as tryptophane, in free

s
t_a

state, have two different singlet electron-stimulated states ^1L_a and ^1L_b; each of which, on principle, may in its own way participate in the photochemical, and hence in the photobiological processes. In other words, the state (condition) of tryptophane residues in protein resembles more the state of tryptophane in the little polar solvent ot polyvinyl alcohol type than in polar solvent of glycerine type, for which, as shown formerly, the entire fluorescence band is conditioned by one type of stimulation of the molecules, the passage (trAnsfer) $^1L_a \rightarrow A$. The polarization spectra of fluorescence in individual proteins do not differ too much between each other (one from the other)$ Only the absolute values of the degree of polarization change within the limits of the mainband and the steepness pf the increase in polarization in the short wave limit of the spectrum. Between these two characteristics of the polarization spectra there exists a definite qualitative dependence: the lower are the degrees of polarization of fluorescence in the main portion of the band, the steeper is the increase in polarization in the short wave portion of the polarizatipn spectrum. Paeticularly sharp rise in the degree of polarization in the short wave part of the spectrum is obserbed in wool keratine - crom 3 to 12%. Marked decrease in the degree of polarization towards the long wave margin of the fluorescence band in such proteins as wool keratine (Fig. 18, b, curve 3) in which the processes of intertryptophane migration of energy are the most effective, may be explained by the fact that in this portion thjere are represented tryptophane residues which are the most

strongly interacting with their microenvironment. Therefore, possessing the lowest situated fluorescence level, these residues are more likely to be acceptors rather than donors, in the processes of intertrypotphane migration of energy.

In the human serum albumine, on the contrary (Fig. 34, a, curve 5_, in the short wave portion of the polarization spectrum the rise is completely absent and throughout the spectrum there are seen positive rates of the degree of polarization about 15% (Bobrovich, Konev, 1965). In the serum albumine, the monochromatic stimulation with light with lambada=296 mmk gives fluorescence spectrum with greatly "cut down" short wave wing as compated to the specter stimulated by 265 mmk, and the difference between these spectra corresponds to the tyrosine fluorescence spectrum. Therefore, in the shortest wave portion of the spectrum of albumine we are actually dealing with fluorescence of tyrosine alone. ~~Therefore, in the shortest wave portion of the albumin spectrum~~ Hence, precisely tyrpsine fluorescence which is depolarized in this albumine (rpte - protein,- as compared to the free state, approximatelt twice as much (instead of 35% we observe 15%), results in straightening out of the polarization spectrum of tryptophane fluorescence in proteins. In other words, in the human serum albumine, along with tyrosine-tryptophane migration there is also ~~ery~~ tyrosine-tyrosine migration of energy with effectivety of the order of 50%.

Protein Phosphorescence

Originally, it was believed that in the phosphorescence spectra of proteins, just as in their luofrescence spectra,

only the tryptophane componenet of luminencence is apparent, whereas the tyrosine and phenylalanine components are extinguished (Vladimirov, Litvin, 1960). However, as a rule, what was correct on the whole, was not observed in a number of individual cases. The tyrosine maximum at 390 mmk in the spectra of low temperature luminescence (77°K) is distinctly marked for a number of proteins, - aldolase, edestin, pepsin and casein (Konev, Bobrovich, 1964) with stimulation of 265 mmk and it diseappears with stimulation of 296 mmk where tyrosine does not absorb.

Fig. 34. Aqueous solution of human serum albumine (a) at t=20°C; 1,2,3 - fluorescence spectra with stimulation by light 265, 253 and 296 mmk reppectively; 4 - differential spectrum, obtained by calculating the fluorescence spectrum with stimulation of with 296 from 265 mmk; 5 - polarization spectrum of fluorescence after emission. Aqueous solution of pepsin (b), t=20°C; 1, 2 - the fluorescence spectra, obtained during stimulat on with 253 or 265 mmk and 296 mmk respectively; 3 - polarisation spectrum after emission

deducting

With total stimulation of the regi n 254-300 mmk, the tyr - sine arm (shoulder) at 385-390 mmk was observed by Burstein, 1964, for egg white albumine and human serum albumin.

In a similar manner, Augenstine and Nag Chaudhuri, 1964, observed in alcohol-dehydrogenase tyrosine arm (shoulder) with stimulation 280 mmk. Fromtheir calculations, 0.5 of the total phsophorescence with stimulation of 280 mmk and 0.7 with stimulation of 240 mmk if conditioned by tyrosine.

In this manner, in a number of proteins, the contribution from the tyrosine component to the ph sphorescence spectrum is quite perceptible and, as a rule, is greater than the contribution from this aminoacid

bution from this aminoacid to the fluorescence spectrum.

According to Longworth, 1961, in the human serum albumine, phosphorescence at 388 mmk, also in lysozym, hemotrypsinogen had a life span of 2.1 ± 0.1 sec. and stimulation spectrum, typical for tyrosine. The remaining portion of the phosphorescence spectrum of these proteins had life span of 5.3 ± 0.1 sec and tryptophane spectrum of stimulation.

The tyrosine componenet of protein phosphorescence is increased under the effect of factors which are responsible for the break of hydrogen bonds, extinguishing the tyrosine fluorescence (Table 10). The thermal denaturation of albumine, casein and edestin increase luminescence at 390-400 mmk. Particularly intensive, well structured tyrosine phosphorescence is observed in proteins under the effect of 8 M lituim iodide. The maximums of the fine structure of phosphorescence of tyrosine residues of a number of proteins of serum albumin, pepsin and others is observed at 354, 366, 387 and 397 mmk, i.e. at the same points of the spectrum as in the free tyrosine (Knev, Bobrovich, 1964). At the same time, the intensity of these maximums represents quite individual characteristic which easily distinguishes proteins among themselves; for example, intensive tyrosine maximums of huma serum albumine are completely absent in the aqueous solutions of hemotrypsinogen.

Phenyalalnine is not apparent in the protein phosphorescence spectra and its stimulation, just as this occurs in the case of fluorescence. Perhaps the only exception may be the mixture of collagen polypeptides, devoid of tryptophane - gelatine, for

which Burstein, 1964, found maximum phosphorescence at 420 mmk, which he ascribed to phenylalanine. Table 10

Position of maximums in low temperature luminescence of proteins and relative intensity of fluorescence (S) and phosphorescence (T)

Protein	Fluorescence lambda max. mmk.	Phosphorescence lambda max. mmk			Relative intensity of luminescence		
					$S_{max.}/T_{400}$	$S_{max.}/T_{440}$	$S_{max.}/T_{460}$
Native hemotrypsinogen	322	412,5	439	450 - 465	4.8	1.8	2.3
heat denaturated	322	413	438	450 - 465	3.0	2.6	3.5
with 8 M urea	322	412	438	450 - 465	5.3	1.8	2.5
" 8 M urea + heat	322	412	438	450 - 465	3.5	1.8	2.4
Native hemotrypsin,	323	412,5	439	450-467	7.0	2.2	3.3
heat denaturated	322	412	439				
with 8 M ures							
heat + 8 M urea							
Native pepsin							
denaturated with heat							
with 8M urea							
Native human serum albumine							
denaturated							
with 8 M urea							
" heat							
Native casein							
heat denaturated							
Native trypsin							
heat denaturated							
with 8 M urea							
" 8 M urea + heat							
Ricin from castor plant							
nztive							
native, stimul. 296 mmk							
denaturated							
heat							
8 M urea							
Native edestin							
native, stim. 296 mmk							
heat denatu ated							
8 M urea							

consult Table, p. 91 for figures

(last column): Native aldolase

Fig. 35. Luminescence spectra at t=77°K, pH 7.4, stimulation 265 mmk; hemotrypsine (1), hemotrypsinogen (2) and amylase (3).

Fig. 36. Luminescence spectra at t=77°K (phosphate buffer pH 7, 4p stimulation 265 mmk) in hemotrypsinogen in native state (1), after denaturation with 8 M urea (2), after denaturation by heat up to 100°C (3)

In this manner, in spite of the remarks made, tryptophane remains the principal, and for proteins, rich in tryptophane, even the only center, responsible for spectral-phosphorescence propterties of proteins in natural state. The phosphorescence, as well as the luofrescence spectra indicate various states of tryptophane in solution and in the polypeptic chain. This is amnifested in the shift of the maximums of phosphorescence spectra by 5-7 mmk. into the long wave side as compared to the free tryptophane in water.

With elimination of the intereffect with the medimum, when the phosphorescence spectrum comes near to the molecular spectrum - the amylase crystals at the temperature of liquid nitrogen - the phosphorescence maximum of protein almost coincide with tryptophane (405, 415, 430, 450 and 480 mmk.). Considerably lesser sensibility of fluorescence as compared with the fluorescence to the effect of the medium will be easier to understand, if we take into consideration that freezing brings closer together the dielectrical constants of various solvents and makes relaxation phenomena impossible. Nevertheless, as one can see from Table 10, even at 77°L there occurs a certain shift into the position of the maximums of phosphorescence with transfer of one protein

into the other. This is particuAarly graphically expressed with the passage 0 - 0 phosphorescence band - mAximum at 410 mmk., which undergoes certain displacements in its position within the limits from 410 (human serum albumine, casein) to 413 mmk (aldolase, trypsine). Denatuzation with heat and 8 M urea results, in,a number of cases, in a certain, alghouth very slight, spectral shifts in phesphorescence (Monev, Bobrovich, 1964).

Therefore, the positions of the maximums of phosphorescence of various proteins always closely coindide among themselves; this indicates small degree of sensitivity of the form of the spectrum of phosphorescence to the influences from the secondary and tertiary structures of the macromolecule.

Quite different is the ratio of the intensity of phosphorescence to the intensity of fluorescence. From Fig 35 one can see, that this ratio for amylase is 6 times higher than in hemotrypsyine; and in the latter, in its turn, it is by 30% higher than in hemotrypsynogen which is close to it in its primary structure. More detailed conception on the extent of variations in the S/T ratio are given in Table 10.

The reconstruction of the secondary and tertiary structures of the protein macromolecules also is accompanied by changes in the relative intensity of ~~fluxxxxx~~ phosphorescence, during heat denaturation (Fig. 36). In those proteins in whichtyrosine phosphorescence is not superimposed on the tryptophane phosphorescence, there takes place a marked relative weakening of phosphorescence; this attains, in case of hemotrypsinogen, 50% as compared to the native state. In contrast to the heat de-

naturation, the 8 M urea denaturation is of little effect on the S/T ratio, for the majority of proteins (Fig. 36, Table 12). Moreover, preliminary denaturation of protein with urea to a certain dergee prevents its changing/due to heat denaturation/ usually

The most probably is the following cause for the high sensitivity of the probability of singlet-triplet radiation passages (transfers?) into the macromolecular organization of protein.

As already mentioned above, the degree of elimination of proton from the nitrogen in the iminogroup has a marked effect of the relative intensity of phosphorescence. If we assume that in this connection, the secondary and tertiary structures of protein create differentmicroenvironment for the tryptophane residues, then the observed changes in the intensity of luminescence may relfect various degrees of entering into the hydrogen bind. In this connection, for instance, in amylase we should accept for the majority of tryptophane residues perfectly free iminogroups, not connected with the neighboring groupings.

Longevity of Protein Phosphorescence

Inclusion of tryptophane into the composition of the macromolecule of protein is little reflected on the life span of phosphorescence. According to the observations of Debye abd Edwards, 1952, the character of extinction of phosphorescence of proteins is determined by two componenes. One of these is extinguished along the exponent - this is the independent luminescence itself from the triplet level. And the other one - is the slower non exponential component, occurring with recombination of the primary products of the photochemical reaction.

According to Longworth, 1961, phosphorescence of the tryptophane component of the human serum albumine, hemotrypsinogen and lysozym is extinguished with tau=5,3 sec., i.e. the same as in the free tryptophane.

Table 11

Duration of stimualted state of phosphorescence ($t_{1/2}$) of certain proteins, lambda or registration 440 mmk, beginning stage of extinction

Protein	$t_{1/2}$ phosphorescence	
	in water	in 8 M urea
Globin hemoglobin	1,89	1.92
Hemotrypsin	2,33	2,52
Hemotrypsinogen	3,23	3,35
pepsin	1,65	1.80
Edestin	2.31	2,30
Trypsin	2,59	-
Casein	1.60	-

Table 11 shows $t_{1/2}$ of phosphorescence of certain proteins from the data of Chernitskii, Konev and Volotovskii. From this Table one can see certain dependence of the $t_{1/2}$ of phosphorescence upon the conformation of the protein molecule which is manifested within a certain diapason of changes of $t_{1/2}$ with the passage (transfer, conversion) of one protein into the other, and changes in the $t_{1/2}$ phosphorescence with destruction of the protein structures with 8 M urea. Moreover, as shown in measurements of Konev and Volotovskii, in individual cases the $t_{1/2}$ of phosphorescence permits to register quite fine structural reconstructions in the macromolecule which occur with the change of the ferment into the maximum active state and which cannot be registered by other methods such as for instance the method

of optical rotation. During conversion from the alkaline solutions of trypsine (pH 10) into solutions with pH 7,5, corresponding to the maximum activity of the ferment, there is observed increased tau of phosphorescence by 50% which is absent in tryptophane and in the biologically inactive states of ferment - preliminary denaturation by alkalinization and the effect of 8 M urea.

As already mentioned above, the tyrosine component of protein phosphorescence has the sam tau as the free tyrpsine: 2,1 sec. in human serum albumine, lysozym, hemotrypsrinogen and insulin (longworth, 1961).

+ Polarization Spectra of Protein Phosphorescence after Ultraviolet-Abdorption and Emission

The vector characteristics of protein phosphorescence haver been studied in our laboratory (Konev, Bobrovich, 1965). Protein phosphorescence as quite low negative rates of the degree of polarization - of the order of 4 + 7%. Such depolarized phosphorescence, as compared to the free state of tryptophane, again indicates intertryptophane migration of energy in proteins. The degree of polarization of phosphorescence remains unchAnged in rproportion to the extinction; this speaks against any possible triplet-triplet migration of the energy. The portion of the extinction curve at which determination of the degree of polarization was done,was isolated wither with the aid of a phosphoscope (intervals of time from 10^{-1} sec. to 10^{-3} sec) or with the aid of optical closing device (stopper, shut-off, bar, shutter) which permits to measure polarization during 0.2-10 sec. after

discontinuation of stimulation.

Fig. 37. 1 - =hosphorescence spectrum of hemotryosinogen in the polyvinyl alcohol film, 2 - phosphorescence spectrum of the fraction of nuclei of white rat liver in polyvinyl alcohol; 3 - polarization spectrum of phosphorescence after emission (hemotrypsinogen); 4 - polarization spectrum of phosphorescence after emission (nuclei), t=196°C. Phosphoscope with permissible time 10^{-3} sec.

In view of the low rates of the degree of polarization, it is difficult to make exact measurements of the polrization spectrum of phosphorescence after absorption. The orientation evaluation shows that it is similar to that of the free tryptophane, but has lesser absolute rates (film of aqueous solution of human serum albumine).

The polarization spectra of phosphorescence of proteins after emission represent a straight line (demonstration of the vibratory structure, again, is difficult because of the low absolute rates), situated below the axis of the abstssa abscissa (Fig. 37). Similar polarizatio spectra are produced also by proteins in its natural physico-chemical surroundings in the composition of nuclei and mitochondria.

All the above permits us to apply the oscillation model of triplet states, obtained for typtophane in the free state, also to the tryptophane residues in protein and in more complex biological structures.

CHAPTER III

MIGRATION AND CONSERVATION OF ENERGY IN HE MACTOMOLECULES OF PROTEIN

The character of the arrangement of the polypeptid chain in space, i.e. the secondary and tertiary structures of protein, facilitate quite close relationship of various chromophores and promotes the appearance of physical intereffects among them. First of all, as result of extremely dense packing of material in the protein globule, conditions are created for migration of energy between the energy isolated chromophores.

Speaking of the processes of migration of energy in the protein systems, one should make a distinction between the intermacromolecular migration of energy and intermolecular migration of energy, i.e. migration of energy bwe between protein and some non protein molecules -prosthetic grouping. Serving as such prosthetic grouping there may be molecules of vitamin, pig ent, coferment, staining agent. The cycle of questions associated with the intermolecular migration of energy has been quite completely reviewed by the authors together with Vladimirov, 1957 1959.

Among the processes of migration of energy of the first tyoe, the leading role, by its effectivity, belongs to the intertryptophane migration of energy. Back in 1957, it has been established that migration of energy between the sulfur-containing aminoacids and tryptphane does not take place (Konev, 1957)

The formerly assumed migration of energy (this author, 1957) from the peptid bonds to the aromatic aminoacids was not later

confirmed)Vladimirov, and Litvin, 1960). For that reason, the delocalization of energy of the electron stimulation in proteions is essentially accomplished with the participation of one aminoacid - tryptophane.

The question of the existence of tryptophane-tryptophane migration of energy in proteins has a rather peculiar history. In 1957, discovering increased intensity of fluorescence in a number of proteins after denaturation, we associated this fact with weakening of migrational extinctioon of fluorescence or tryptophane in the denaturated protein, as a result of a certain loosening of the macromolecules and increase of the average distance between the residues of this aminoacid. It would seem that these conjectures could be confirmed by the experiments of Vladimirov, 1957 in which he registered concentrational extinction of fluorescence in aqueous solutions of tryptophane. However, as we shall see below, changes of the quantum output of fluorescence in tryptophane cannot be associated with the migration of energy.

It is quite clear that with the aid of scalar spectral characteristics it is difficult øto notice the fact of spacial disconnection of the center which absorbed the light, and the center which emitted quantum luminescence for the completely identical molecule of the donor and the acceptor. In this case, study of the vector characteristic may aid in the registration of the process of migration of energy - the polarization of fluorescence. In the given case, the migration of energy will be manifested in the non cpincidence of orientation of

the absorbed abd the emitting light tryptophane molecules, i.e. in the effect of depolarization of fluorescence.

The degrees if polarization of fluorescence of the free tryptophane and proteins were measured by Weber, 1960, and independetly from him by Konev and Katibnikov, 1961. Stimulating fluorescence by natural no polarized light, and working with fine (30 mmk.) layers of tryptophane and proteins in propylene glycol at the temperature of -70°C, Weber found that for 265 mmk. the degree of polarization of tryptophane fluorescence is 15%, whereas for proteins it does not exceed 6-8%. With recalculating to linear non polarized stimulating light, the figures increase to 26% and 10-15% respectively. Konev and Katibnikov, 1961, using linear polarized light for stimulation, obtained P_{265}=25% for gkycerine solution of tryptophane and 2-7% for the structural proteins of the type of keratine of various prigins (sheep wool, calf fur, axis of chicek feather).

Some not complex calculations and rationalization permit us to discard the possibility of depolarization of fluorescence at the expense of secondary effects - effects of light dissemination and secondary luminescnce. For example, the effect of depolarization is preserved even in the fine layers of protein where dissemination is practically absent.

More serious contradiction against the unequivocal conclusion from the fact of depolarization of fluorescence as to the migration of energy could be drawn from the possible changes in the degree of symmetry of oscillator of tryptophane radiation, included into the protein content. If we assume that the

oscillator of tryptophane fluorescence changes from the linear to a fully or partially flat one, then this in itself should produce the depolarization effect. Such a possibility has been contemplated by Konev and Katibnikov, 196[illegible], and they discarded it, because with orientation of tryptophane residues in proteins of the type of wool keratine or seriosine of silk they obtained greatly polarized fluorescence (up to 30-50%).

However, in 1960, Weber mentioned still another possibility for depplarization of fluorescence in proteins, not associated with the processes of migration of energy. Webers reasoning was as follows.

As already mentioned above several times, the long wave band of absorption of tryptophane at 280 mmk conceals in itself two electron passages (conversions, transfers), oriented to each other at a angle close to 90°. At the same time, the absorption spectrum of the negative oscillator at 289 mmk is inscribed into tje circle of a wider soectrum of absorption of the positive oscillator. Both these oscillators of absorption light up the energy essential through the one and the same ^1L_a fluorescence oscillator, coinciding in direction with the positive absorption oscillator ^1L_a. Owing to this, the degree of fluorescence polarization, actually depending upon the Angle between the absorption oscillator and the radiation oscillator, depends also upon the percentage portion of the quanta of absorption of the positive positive rates in degree of fluorescence) and "negative" (negative rates of degree of polarization of fluorescence of the oscillator. If, for example,

the absorption of the "negative" oscillator, for some reason whatsoever, increases, then this alone without any participation of migration must result in decreased positive values of the degree of polarization of total fluorescence, i.e. the effect of depolarization. Based on these considerations, Weber assumed that inclusion of tryptophaner into the polypeptid chain precisely is accompanied by such changes in the tryptophane oscillator system, with which a greater amount of absorbed quanta is to be attributed to the negative oscillator.

Thiks original hypothesis of Weber's was changed to a certainty in 1961, based on the following fact. It was found that human serum albumine fluorescence, containing only one tryptophane residue, is likewise depolarized as compared to the free tryptophane. Of course, in this albumine, the intertryptophane migration is absent and it remained to admit that the inclusion of tryptophane residues into the composition of the albumine structure results in relative increase in power of the "negative" oscillator and, consequently, decrease in the degree of polarization. This fact seriously undermined the possibility of migration interpretation also in other albumines.

A second weighty argument, permitting Weber to contradict the intertryptophane migration of energy in proteins, was in White's data, 1960, on the acid titration of fluorescence in the oxen blood serum albumine. This albumine, containing two tryoptophane residues, also has two steps in acid titration of fluorescence - at pH 0,5 and 3,5. The first of these steps is associated with the tryptophane residue in which its own carboxyl group is blocked by entering into the peptid bond, the second tryptophane residue is in the neighborhood of the carboxyl group of asparagic or glutaminic acid. The conversion of the carboxyl groups of dicarbon aminoacids during oxidation from the

COO^- state into the COOH is accompanied by extinction of tryptophane fluorescence. This is how Weber reasoned. If there were an active migration between the tryptophane residues, then, extinguishing the fluorescence of one of them, we thereby would extinguish also the fluorescence of the second, inasmuch as energy then would migrate from it into the first residue and here become expended in heat. Therefore, in the case of migration of energy, we should observe only one step of acid tritraion. But actually, there are two of them, and this speaks against the intertryptophane migration of energy.

These, breifly, are Weber's arguments.

Let us analyse carfully all proofs, both in favor of and against the migration of energy in proteins, and review the mechanism of depolarization of protein fluorescence.

One of the basic differences between the state of tryptophane in aqueous solution and in the protein molecule is the greater hydrophobic condition of the microenvironment (lesser polarity), in the latter case. In this connection, it is natural to suspect that this decrease in the medium polrization might not be accompanied by increased relative cpntribution of absorption in the passage (transfer) ^1L_b? An Answer to this question may be found in comparison of polarization spectra of fluorescence of tryptophane after absorption in various viscous solvents. Inasmuch as the "negative" oscillator has a clearly marked maximum of abosrption at 289 mmk., then its increased contribution to absorption must be expressed in deepening of the gap in polarization spectra with this length of wave. However, experimental data contradict such an evantuality. The gaps in the polarization spectra in tryptophane and indol in glycerine (strongly polarized medium), sugar rock candy (average polarization) and

polyvinyl alcohol film (weakly polarized medium) were found to be practically identical (Chernitskii, Konev, 1964) (Table 12).

Moreover, the polarization spectra of fluorescence of proteins proper has even somewhat less marked gap at 289 mmk., than in free tryptophane. In good concurrence with this conclusion is also the circumstance that, in the protein absorption spectra, the ratio of extinction at 280 and 290 mmk does not change toward the long wave absorption, as compared with the free tryptophane. In other words, the electron transfer (passage) ^1L_b of tryptophane in protein does not perceptibly increase, as compared to tryptophane in free state. This evidently contradicts Weber's assumption o the relative increase in absorption of the negative oscillator.

The fact that inclusion of tryptophane into the peptid bond is not accompanied by noticeable changes in the oscillatory nature of tryptophane, is also confirmed by identical degrees of polarization of tryptophane fluorescene and its dipeptid glycyl-tryptophane (Fig. 18, a, curves 3 and 4). W weak influence on the polarization spectra is exerted, apparently, also more remote groups of protein than the nearest peptid bond. This may be condlued from the experiments in which the disintegration of the secondary and tertiary structures of the protein macromolecules, during heat denaturation, and dioxane denatiration, is not accompanied by changes in the form of polarization spectra (Konev, Chernitskii, Bobrovich, 1964).

In this manner, it is possible to enumerate a number of arguments, showing that the changes in the oscillator model of tryptophane residues cannot explain the phenomenon of depolarization of protein fluorescense, as compared to free tryptophane. And, conversely, it is possible to

cite a number of proof that the depolarization fluorescence of protein has a migrationary nature. The most direct proof of the intertryptophane migration of energy in protein can be derived from the polarization spectra of fluorescence after emission of proteins (Konev, Borovich, Chernitskii, 1964). This possibility is associated, first of all, with the fact that the intertruptophane migration of energy, as these authors found it, in model conditions, causes quite characteristic changes in the form of polarization spectrum of fluorescence after emission (radiation) In proportion to the increase in tryptophane concentration in the polyvinyl alcohol film and, thereby, strengthening of the processes of intertryptophane migration of energy, the concentrational depolarization takes place differently in various points of the radiation spectrum: the degree of polarization in the long wave portion of the spectrum falls quite markedly, whereas the shortest wave margin of the spectrum is depolarized very little (Fig. 18 a). With high concentrations of tryptophane, the course of polarization spectrum becomes opposite to the course of the spectrum in case of low concentrations: instead of the usual decrease in the degree of polarization in the short wave portion of the spectrum, there is seen its increase. What is it that conditions such a change in the polarization spectrum in proportion to the intensification of the processes of energy migration?

Table 12

The ratio $K=\frac{P_{270}}{P_{289}}$ for tryptophane and indol in various solvents

	$K=\frac{P_{270}}{P_{289}}$	
continued	tryptophane	indol

cont. Table 12

Médium	tryptophane	indol
Glycerine (room t^{o})	1,3	-
Propylene-glycol (t^{o} -7o^{o}C)	1,5	2,1
Sugar candy (room t^{o})	1,3	2,5
Film of polyvinyl alcohol (t^{o} of the room)	1,3	2,1

The observed changes in the form of polarization spectra are based onthe following processes. As mentioned before, the negative oscillator conditions only insignificant portion of tryptophane luminescence in the polyvinyl alcohol. Transfer of the major portion of the absorbed energy to the positive oscilla or is, essentially, equivalent, to the extinction of its own fluorescence. A direct consequence of extinction should be shortened span of life of the stimulated state $^{1}L_{b}$.

Inasmuch as the effectivity of the energy migration depends upon the life span of the stimulated state, depolarization must decrease in proportion to shortening of this time, i.e. in the region of its own luminescnce by oscillator $^{1}L_{b}$ depolrization should be very slight.

As one can see from Fig. 18,a, this is what is actually observed.

In this manner, from the form of the polarization spectrum of fluorescence after emission one can make a judgment (or evaluate) on the course of energy migration processes. Now, it remains to compare the polarization spectra of fluorescene of proteins with the similar spectra, obtained with concentrated films of polyvinyl alcohol. The comparison of the polarization spectra of protein fluorescence after amission (Fig. 18,a) shows that they coincide with the similar spectra of

tryptophane, under the conditions of effective energy migration. There should not be any such coincidence, were the depolarization of protein fluorescence caused by another reason than depolarization of fluorescence of concentrated solutions of tryptophane, i.e. not by energy migration, but by increased absortion [1] Lb. In the latter case, decreased degree of polarization in the long wave portion of the spectrum would be compensated for by its growth in the short wave portion. This, however, is not really observed, and with lambda=300 mmk, the degree of polarization of fluorescence of protein is always lower than in non concentrated solutions of tryptophane.

In view of the importance of the conclusion, let us analyse the polarization spectra of fluorescence of proteins after emission somewhat in greater detail.

Inasmuch as making models of the state of tryp ophane residues in protein by means of tryptophane molecules, under various conditions, is only rational in the case when the entire band of protein fluorescence is really conditioned by tryptophane alone, then it would be necessary to compare the fluorescence spectra of protein solutions, stimulated by various wave lengths of monochromatic light. The complete identity of these spectra, during stimulation with monochromatic light of any wabe length in the interval 250-300 mmk leAves no doubt that the only center of luminescence in these proteins is actually tryptophane (Fig. 28). As a matter of fact, the presence of oyher centers of luminescence, besides tryptophane, would inevitably result in changing of the form of the fluorescence spectrum, for example, with such wave length of stimulation which correspond to the maximum absorption of this other center (tyrosine, phenylalalnine).

A second possible, non migration, cause of changes in the polarization spectrum of fluorescence after emission of proteins is the superposition on the primary fluorescence of secondary, tertiary and nigher order fluorescences. If there were actually registered a mixture of luminescence of various orders, then in the shortest wave portion of the spectrum of fluorescence, the contribution of the secondary fluorescence would be maximum as compared to the entire long wave portion of the spectrum. The effect of re-Absorption of fluorescence, taking the quanta only from the whort wave reabsorbed portion of the spectrum, would thereby cause a quick decrease in the ratio of intensity of the primary and secondary fluorescence, in proportion to approaching the short wave margin in the fluorescence band and, therefore, to the effectof complete depolarization,

But, actually the polarization spectra of protein fluorescence after emission show exactly the opposite ratio: polarization towards the short wave margin of the fluorescence band increases. Moreover, the most elementary calculation shows that we can practially disregard the fluorescence of second and higher orders, even for such concnetrations of protein where there is complete absorption of light in the maximum 280 mmk. Comparison of the the absorption spectra and fluorescence spectra sh ows that under these conditions there is reabsorbed not more than 5% of the quanta of fluorescence. The intensity of fluorescence can be obtained in the following equation: $I=(1-\beta+\beta\varphi 1-\beta+\beta^2\varphi^2(1-\beta)^t$ etc., where beta is the precentage of absorption o the primary fluorescence in the tested solution; φ is the quantum output of fluorescence; 1 - beta -inten-

sity of primary fluorescence, reaching the detector; $\beta\varphi$ is the intensity of the secondary fluorescence; $\beta\varphi(1-\beta)$ is $_2$ intensity $_2$ of the secondary fluorescemce, reaching the detector; $\beta\varphi$ (1 - beta) is intensity of the tertiary fluorescence, reaching the detector, etc.

Taking φ to be equal to 0,2, beta equal to 0.05m we obtain the intensity of the secondary fluorescence, equal to 1% of intensity of the primary fkuorescence.

Moreover, the characteristic form of the polarization spectra of fluorescence remains preserved unchaged even in such fine and diluted layers of protein in which the processes of reabsorption are absent (optical density even in the maximum of absorption at 280 mmk is 0.05, i.e. about 5%). The unchanged form of the polarization spectra of fluorescence after emission in the wide mArgins from $D_{280} = \infty$ to $D_{280} = 0.01$, is the best proof of the true nature of the registered spectral ratios.

In this manner, the possibility of presenting "models" of polarization spectra of fluorescence in tryptophane residues of protein with similar spectra of free tryptophane, being under the conditions of effective energy migration, presents a strong argument in favor of the processes of energy migration in proteins. Supplementary proofs of the existence of processes of intertrop ane migration of energy may be obtained from the the polarization phosphorescence of proteins. The use of the degree of pokarization of protein phosphorescence as a sign of energy migration is of great interest from two points of view First of all, because the phosphorescence is shifted by more than 100 mmk into the long wave side, as compared to fluorescence, and, therefore, in the given case, the secondary effect

of light disersion is greatly diminished. And, in the second place, and chiefly because, as it has been mentioned in the preceding chapter, the oscillator of tryptophane phosphorescence is oriented practically perpendicular to the plane of the indol ring, i.e. the phosphorescence oscillator is perpendicular to the oscillator ^1L_a, and also to the oscillator ^1L_b. Consequently, changes in the relative contributions into the absorption of the oscillators should not be reflected on the degree of polarization of phosphorescence. And, conversely, the processes of energy migration must in equal degree depolarize, both the fluorescence and the phosphorescence of proteins as compared to tryptophane.

Measurement of polarized spectra of phosphorescence of proteins after absorption and emission shows that it is depolarized as well as is the fluorescence (Fig. 37).

Thus, proteins have rates of the phosphorescence polarization degree of the order of -5 - 7%, i.e. approximately twice lower than in tryptophane itself (-13 -15%). Protein fluorescence is also depolarized in the same degree, Therefore, experimentally, exclusing possible depolarizing influence of the negative oscillator of absorption, there is still registered considerable depolarization which, for that reason, may only be caused by migration of energy.

Finally, a third main experimental method which permits selection between the "migrational" and "oscillating" mechanism of depolarization, consists of the fAct that the relative rol in the absorption of oscillators is manifested practically instantaneously, at the moment of absorption of light, whereas migration of energy, occurring throughout the entire period of time of the stimulated state, must

be accompanied by the effect of depolarization of fluorescence developing in time. At the same time, polarization of fluorescence in the molecules, remaining in the stimulated state the shortest period of time, must be ~~thexhighe~~higher than that in the molecules with the longest period of life: polarization must decrease in proportion to the extinction of fluorescence. As a conseaquence of this, with gradual ~~xxxxxx~~ extinction of fluorescence, removing first of all fluorescnence of "long-living" molecules and then later more and more "short-living" molecules, there should be increased degree of polarization of phosphorescence in the case, when the initial depolarization was caused by energy migration. But if depolarization is caused by changes in the portion of absorption by the oscillators, then such a ratio, naturally, will not be observed and the degree of fluorescence polarization will remain constant in proportion to the extinction of fluorescence.

As we know, S.I. Vavilov considered depolarized luminescnce as one of the most direct proofs of the existence of energy migration.

From Fig. 38 one can see that twenty-times repeated extinction of casein fluorescence with fluorescein (hard films of casein with fluorescenin) is accompanied by increased degree of polarization of fluorescence from 10 to 15-16%. At the same time, the casein films with greatly extinguished fluorescence required two important conditions: the trophane portion of the fluorescence spectrum is not distorted and there is observed independence of this portion of fluorescence spectrum from the length of wave in the stimulating light. At the same time, the increase in protein fluorescence polarisation occurs uniformly throughout the entire long wave portion of the

spectrum of fluorescence, as demonstrated in the illustrated fluorescence spectra after emission (Fig. 38). This experiment may be considered as crucial (experimentum crucis) which unequivocally proves energy migration in proteins, inasmuch as one cannot explain by any other causes (secondary effects, changes in the oscillation model, etc.) the increase in the degree of polarisation in proportion of shortened period of life span of luminescent centers. The possibility of rotation depolarization in long living molecules, then, is completely excluded -- the time of relaxation of proteins even in the aqueous solutions is 20-50 times greater than the life period of the stimulated state, not to mention the films in which the difference between these periods of time may be hundred thousand times. A direct experimental confirmation of this may be found in the coincidence of the rates of degree of polarization of protein solutions in water and in glycerine at room temperature, in hard (solid) protein films (viscosity is equi-infinite) at room temperature and at the temperature of liquid nitrogen.

Fig. 38. Casein films proper. t=20°C, stimulation 265 mmk. Fluorescence spectra without extinguisher (1) and with extinction 20 times with fluorescenin (2). Polarization spectra of fluorescence after emission on non extinguished (3) and extinguished 20 times (4) casein.

Fig. 39. Globin fluorescence spectra (1) and hemoglobin fluorescence spectra (2), stimulation lambda=265 mmk; globin (3) and hemogrlobin (4) phosphorescence spectra in 2M Lij,t=-196°C. stimulation lambda=265 mmk (room temperature,aqueous solutions).
Polarization spectra of fluorescence after emission in globin (5) and hemoglobin (6), stimulation lambda=265 mmk, room temperature. Polarization spectra of fluorescence after emission in globin (7) and hemoglobin (8), registration lambda=340 mmk., room temperature.

One of the most graphic proofs of the existence of intertry ptophane energy migration in proteins was obtained in the example of hemoglobin (Konev, Bobrovich, Chernitskii, 1965). As we know, owing to the effective transfer of energy from tryptophane to heme, the tryptophane fluorescence is extinguished: the quantum output is, from our data, 0,5%, after Teale, 1959, less than 0,2%. Therefore, the energy of stimulation, not transferred to the heme, will be realized in luminescence with extremely short duration, $tau_1 = \frac{tau_0 B_1}{B_0} = \frac{3,0 \cdot 10^{-9} sec. \cdot 0,005}{0,14} \approx 10^{-10} sec.$, where tau_1 and tau_0 are life durations, and B_1 and B_0 are the quantum outputs of hemoglobin and globin quantum outputs of fluorescences, respectively. In other words, the stimulated states of tryptophane which succeeded in avoiding participation in the ghighly effective process of tryptophane-heme migration, could the more be capable of escaping considerably much slower process of intertryptophane migration. Actually, measurements of the polarization spectra of fluorescence of hemoglobin and its protein portion - globin - have completely confirmed this assumption. The globin of hemoglobin has typical protein polarization spectra abfer absorption and emission with considerable polarization P_{265}^{350} of the order of 9-10% (Fig. 39). The addition of the heme groupings does not change the long wave profile of luminescence and the position of the maximum, however, it considerably strengthens the short wave portion of the spectrum; and this, once again, confirms that the fluorescence is formed by long duration ^1L_a A luminescence, undergoing effective extinction, and short-lived ^1L_b A luminesence, less extinguished by the joined here (Fig. 39). Simultaneously the portion of oscillator ^1L_a luminescence, remaining after extinction,

gives high rates of polarization degree. The polarization spectra of fluorescence after absorption and emission in hemoglobin are more than twice as high along the axis of the coordinate, as compared to globin, so that P_{265}^{350} of hemoglobin becomes equal to 20%, i.e. it acquires rates, characterstical for tryptophane in the free state. Fluorescence spectrum taken through two monochromators and two polarizors, under the same geometrical conditions, as for the polarizati n measurements, shows that no extraneous luminescence is supreimposed on the tryptophane luminescence.

Similar in theory proofs, in favor of the existence of intertryptophane energy migration in proteins may be obtained in studying the ratio of the polarization or fluorescence degree and the temperature. As we know, (Levshin, 1951), the temperature extinction of fluorescence in organic molecules is accompanied by shortening of the time of life of the FLUORESCENT STATE$ This, in turn, should decrease the probability of energy migration. Actually, Feofilov, 1960, observed increase in the degree of polarization of fluorescence of concentrated rock candy dyestuff at elevated temperature. Based on this, one would expect that with exclusion of possible kinetic re-orientation of the tryptophane residues, during the time of the stimulated state, a rise in temperature on the plus side, should result in increased polarization, accompanying the effect of temperature extinction of proteins.

Serving as main objects for such investigation, we selected two proteins - wool keratin and silk fibrion. This choice was justified by the following (causes) moments. First, both keratin and fibrion represent solid (hard, firm) specimens, in which at any rational temperature, any possible relaxation of tryptophane residues during the time of

stimulated state is completely excluded. Second, the fluorescence of these proteins is the most depolarized, as result of which the effect of the increase in degree of polarization of fluorescence with elevation of temperature must appear to be the most contrasting.

Before considering the polarization measurements, it was necessary to be convinced that extinction of fluorescence in the selected objects actually exists and that it may be referred to extinction of the second type.

The dependence curves $I_{fl}=f(T)$ for wool keratin and silk fibrion are given in Fig. 40. From this figure, one can see that heating from 20° to 90°C is accompanied by a monotonous drop in fluorescence intensity. At the temperature of 90°C fluorescence intensity weakens about 20 times. The fact that the extinction has a physical, rather than chemical nature (for example, thermal oxidation of tryptophane) is proved by the reversibility of extinction: with the reverse lowering of temperature the intensity of fluorescence assumes initial rates at all points, including the starting point (20°C). This indicates absence of chemical changes in tryptophane within the tested diapason of temperature. With heating up to higher temperatures (100-130°C), there is observed hysteresis between the direct and reverse branches of temperature "titration" and even with the naked eye one can see marked yellowing of the specimens. In other words, at temperatures above 100°C there takes place chemical thermodestruction of tryptophane. In order to avoid complicating interpretation of facts, we have limited ourselves, in this connection, to the upper temperature threshold of 90°C.

The chemical identity of the tryptophane molecules at 20° and 90°

90°C, may be demonstrated by taking protein fluorescence spectra at these temperatures. As one can see from Fig. 41 and 42, heating is accompanied only by little widening of the spectra without shifting of the position of their maximums.

Fig. 40. Ratio of degree of polarization P$^{340}_{265}$ (1) and intensity of fluorescence (2) in silk fibrion and temperature; 3 and 4 - the same, for wool keratin.

All this permits us to classify keratin wool extinction and silk fibrion extinction as extinction of second class.

Fig. 40 gives results of measurements of degrees of fluorescence polarization at various temperatures of the specimens. From Fig. 40 one can see that with elevation of temperature from 20° to 90°C, the degree of fluorescence polarization gradually increases from 5-6% to 10#12%. The increase in the degree of polarization, as well as the fluorescence extinction, are completely reversible.

The wool keratin polarization spectra of fluorescence (Fig.41) and that of silk fibrion (Fig.42) at temperatures of 20° And 90°C have a typical tryptoprophane form. They differ one from the other at all points only by the absolute rates of the degree of polarization. The illustrated curves show that the effect of protein fluorescence depolarization develops in time, i.e. it has a migrational nature,

Finally, a few words about another method for proving the inter-tryptophane migration of energy in proteins. If, by any means whatsoever, we decrease the number of tryptophane residues in mAcrocolecule of protein, which on the average contain only one tryptophane residue, then this should result in discontinuance of migration and, therefore, to increase in

to increase in the degree of fluorescence polarization.

Actually, with gradual photochemical destruction of tryptophane residues in casein during radiation of protein solution with ultra violet light with lambda=250-300 mmk. there is observed a rise in the degree if fluorescence polarization from 10 to 18%. The experimental curve of the ratio of the degree of polarization to the degree or destruction of the tryptophane residues P=f(1 fl. casein)=f(t of radiation) coincided with the theoretically calculated curve, according to the law of addition of degrees of polarization of two first luminescent forms of casein molecules: casein 2 of tryprophane residues $\xrightarrow{h\nu}$ casein 1 tryprophane residue $\xrightarrow{h\nu}$ casein 0 tryptophane residue. Preliminarily concentrations of the first and second luminescent forms of protein are calculated for any moment of time since the start of radiation. The coincidence (concordance, agreement) of the theoretical and expe imental curves was observed in the case, when the degree of polarization of the second form (tryptophane in protein, but there is no energy migration) was equal to 22%. In other words, the tryptophane residues in protein after exclusion of energy migration have fluorescence polarization degrees very close to those of tryptophane in the free state.

Fig. 41. Polarization fluorescence spectrum after emission (1) and fluorescence spectrum (4) of wool keratin at t=20°C, lambda of stimulation 265 mmk; 2 and 3 - the same, for temperature of 100°C.

In this manner, all the combined fats reviewed above, incontestably testify in favor of the processes of energy migration in the protein macromolecules. There arises the natural question, then, how we can

correlate these facts with the facts, mentioned by Weber, and first of all with the fact of depolarization of human serum albumine depolarization which onyl contains one tryptophane residue. Indeed, in that case, there is no ~~interetryphane~~ intertryptophane energy migration, while fluorescence nevertheless is depolarized. Hence, the idea that only the inclusion of tryptophane residues into the composition of the protein macromolecules, without any migration, is sufficient to make its fluorescence depolarized.

Fig. 42. Polarization spectrum of fluorescence after emission (1) and d fluorescence spectrum (4) of silk fibrion at t=20°C, lambda of stimulation 265 mmk; 2 and 3 - the same, for temperature of 100°C.

\# In order to better analyse the mechanism of serum albumin fluorescence depolarization, let us first of all direct our attention to the fact that Teale and Weber, 1960, 1961, assumed too absolutely the impossibility of energy migrztion from tyrosine to tryptophane.

The concenption of the impossibility of energy migration from tyrosine to tryptophane is based on several facts. First of all, in the stimulation fluorescence spectrum of serum albumine, in the region of absorption of tyrosine residues there is observed not sensibilization of tryptophane fluorescence, but, on the contrary, screening. Overlooking other proofs, let us first of all turn our attention to the fact that actually this screenin is far from complete and, apparently, it is more correct to say that the basic portion of tryosine molecules performs functions of pure screen, while the other portion of molecules still represents sensibilizator. This follows, most of all, from the experimental data of Teale himself on the

on the determination of quantum output of fluorescence of human serum albumine with various lengths of waves of stimulation. Thus, with the wave length of 300 mmk, where absorption is conditioned exclusively by tryptophane, the quantum output of protein is the same, as in tryptophane in free state in aqueous solution., - 0.21. At 280 mmk., when the tryptophane absorption portion is 19,5% quanta and 80,5 is xxx tyrosine absortion, the quantum output of tryptophane fluorescence of albumine must in the case of pure screening drop down to 0.04. But actually, the quantum output of serum albumin fluorescence at 280 mmk. only falls to 0.07. In other words, tryosine represents both the screen and the sensibilizaoor and of each seven radiated by tryptophane quanta three quanta appear at the expense of energy migration from tyrosine residues. Therefore, in the case of serum albumine, the single depolarization fluorescence of the only tryptophane residue may be naturally explained by the xxxm tyrosine-trhyptophane energy migration. Such an explanation also permits qualitative verification (Bobrovich, Konev, 1965). With stimulation of 300 mmk., when tyrosine does not absorb and tyrosine-tryptopahne energy migration is absent, the degrees of tryptophane fluorescence polarization and that of serum albumine must be identical. Actually, the degrees of serum albumine and tryptophane polarization fluorescnce are respectively 32% and 35% (Fig. 32). Inasmuch as fluorescene, appearing at the expense of transfer of energy, is depolarized, it was possible to evaluate the depolarization, provoked by energy migration from mi tyrosine, at 280 mmk., based on the following formula

$$\frac{P}{P_o} = \frac{I_o}{\sum I_k}$$

where P is fluorescence polarization in case of energy migration; P_o is fluorescence polarization in the absence of energy migration; I_o is intensity of molecule luminescnce, radiated without transfer of energy; $\sum I_k$ is the total intensity of fluorescence (Galanin, 1955).

As explained above, for 280 mmk $\frac{I_o}{\sum I_k}$ is equal to 4/7. Now it is simple to calculate the degree of fluorescence polarization of tryptophane in this the case, when tyrosine-tryotophane energy migration is absent. The degrees of polarization will be equal to $P_o = \frac{\sum I_k}{I_o} P = \frac{7}{4} 0,14 = 0,25$, i.e. the same as in tryptophane oxx itself in the free state for this wave length. Consequently, with introduction of correction for depolarization, conditioned by tyrosine-tryptophane energy migration, the polarizational spectra of protein fluorescence and the free tryptophane become identical.

In this manner, tryptophane residues, included in the composition of the protein macromolecule, do not essentially change their oscillator nature and the very influence of the peptid bond or the macro-environment is incapable of lowering the degree of polarization, as this is easily accomplished by tryptophane-tryptophane migration of energy. Now the example of serum albumine no longer appears as a fact, contradictory to the tryptophane-tryptophane energy migration, but on the contrary, as a fact, indirectly (circumstantially) confirming it. Likewise, the second consideration, mentioned by Weber, does not contradict the inter-tryptophane energy migration; precisely, the two steps in the curve of the acid titration of oxen serum albumine fluorescence. The two steps only indicate that the constant of the speed of energy migration of "neutral tryptophane"-"acid tryptophane"

is smaller than the constant of the speed of fluorescence path of deactivation of the tryptophane molecule.

To sum up, the combination of the facts, exposed above, leads to the conclusion that inside of the macromolecule of protein there occur processes of transfer of energy of electron stimulation between the residues of tryptophane. As shown earlier, the tryptophane residues may be found in protein in three different electron-stimulated states which correspond to the electron levels of energy: ^1L_a and ^1L_b for the singlet stimulated states and ^3L_a for the triplet. In this connection, the first question in the explanation of the mechanism of the intertryptophane migration of energy must be as follows: between which electron levels of the stimulated molecule does the energy transfer occur? Do all the electron levels participate in the energy migration, and if so, in what proportion?

Polarizational measurements make us discard the possibility of participation in the processes of migration of energy for the singlet level ^1L_b and the triplet level ^3L_a. The first follows from the fact that the molecules which are in the electron-stimulated state in ^1L_b hardly undergo at all the effect of depolarization of fluorescence in proportion to the increase in tryptophane concentration. As mentioned before, the portion of the tryptophane fluorescence spectrum and protein spectrum, corresponding to its own luminescence ^1L_b, is little depolarized with the increase in tryptophane concentration in the solid solution of polyvinyl,alcohol. This is illustrated in Fig. 18.

In his time, Weber, 1960, 1961, while studying the polarization spectrum of indol fluorescence in the region of concentration de-

polarization, arrived at the opposite conclusion: energy migration occurs only with the participation of the electron level ^1L_b, the stimulation of the band ^1L_a is not effective for the energy migration." It may be shown that this conclusion of Weber's not only does follow, but actually contradicts the experimental data. From the data, as given by Weber himself for indol, first of all, it follows that with the increase of concentration of solutions from 0,01 M to 0,2 m/l the fluorescence is depolarized in the region of stimulation 260-265 mmk 2.8 times, i.e. more than in the region 290 mmk (2,3 times). But in reality, based on Weber's premise, we should expect the opposite:migration occurs only with the participation of ^1L_b, absorbing at 290 mmk., and, therefore, it is maximum with this wave length, and this should be followed not by drop but by a rise in the degree of polarization, since migration in this case depolarizes the "negative" fluorescence, lowering the positive rates of the degree of total fluorescence polarization. The second experimental fact, mentioned in Weber's work, consists in that, in the concentrated solutions of phenol with admixture of indol (1 M phenol + 0,01 indol and 1 M phenol + 0,05 indol) in the region of stimulated phenol (270-275 mmk) there is observed the greatest negative rate of indol fluorescence polarization - on the order of 1 - 2%. This Weber interprets in this manner: as a result of predominence of indirect stimulation of infol by energy, migrating from phenol, and due to the fact that in migration only the negative oscillator is involved, then in the experiment the negative degree of polarization is apparent. But actually, we know well that eben the very act of migration itself (migration of energy between the chaotically arranged molecules) out-

side of dependence upon the oscillation nature, must be accompanied by practically complete depolarization fluorescence (Galanin, 1956); even Weber's own data rather speak for only the zero polarization, but never the nergative one.

Finally, the third and the only, it would seem, weighty argument is the absence in Weber's curves, of concentration depolarization at the points 300 - 310 mmk. Actually, inasmuch as at these points only the oscillator ^1L_a ia stimulated in pure form, then at first glance one might think it is inactive for the process of energy migration.

However, we have already shown in the foregoing chapter, that frequently fluorescence with stimulation 300 mmk and over it not depolarized, becAuse in this region there is considerable absorption of admixtures which due to their low concentration as compared to rhe basic form of tryptophane, naturally, do not undergo depolarization. Fluorescence of the spectrally pure proteins is depolarized at 300 mmk as well as at 265 mmk.

In this manner, Weber's concenptions o the inactivity of the oscillator ^1L_a in the migration of energy are devoid of any serious experimental foundation.

In spite of the fact that the triplet-triplet energy migration with the exchange-resonance mechanism, has been shown in many substances (Terenin, Ermolaev, 1954; Ermolaev, 1964) and that even at room temperature proteins has a marked stationary concentration of molecules in the triplet state ^3L_a, - in spite of all this, - we shuld likewise discard the idea of hte possible energy migration during the time of presence in the phosphorescent, triplet state. This is first of all suggested by the identity of the migration

depolarization fluorescence and phosphorescence of protein in hemotrypsinogen, as compared to the free tryptophane. For this protein $\frac{P \text{ tryptophane}}{P \text{ protein}}$ is equal to 2,8 for fluorescence and 2,7 for phosphorescence. The closeness of the found ratios directly indicates that during the time of the stay of the tryptophane molecule in the ff triplet state, there takes place no additional energy migration which would have been accompanied also by additional depolarization. The same is indicated by the constancy of the degree of phosphorescence polarization at various stages of its extinction. The degrees of phosphorescence polarization at different speed os phosphorescence (interval of the curve of extinction 10^{-1} - 10^{-3} sec.), as well as with the use of the mechanical shut-off (interval of the cuve of extinction 10^{-1} - 10 sec.) proved to be identixal (Konev, Bobrovich, 1964). Therefore, the entire transfer of energy is accomplished during the time of life of the singlet stimulation state ^1L_a. This is well confirmed by the concentrational tryptophane fluorescence depolarization, preciselt in this region, where radiates the oscillator ^1L_a, as one can see from the polarization fluorescence spectra after emission (Konev, Bobrovich, Chernitskii, 1964).

Therefore, the electron level ^1L_a participates in the energy migration. Then what is the physical mechanis of this transfer of energy?

The absence of any need in structural factors and the possibility of the energy migration process between the tryptophane molecules in solution and in the polyvinyl alcohol film, both in native and in the denaturated proetin molecule, excludes that participation in the transfer of zone

in the transfer, of zone conductivity or exciton.

At the same time, all the basic conditions for the process of energy migration with the mechanism of induction resonance (Vladimirov, Konev, 1957) are manifested: tryptophane possesses luminescence, the spetra-of spectra of fluorescence and absorption of molecules, althoug slightly, but definitely coincide, the tryptophane residues in protein are separated by small distance of the order of 10-30 Å.

It is possible to arrive at a conclusion on the energy migration mechAnism also based on the characterof the ratio of probability (speed) of energy migration from the distance between the donor and the acceptor of the energy. The theory of energy migration with the mechanism of induction resonance, developed by Foerster, 1964, 1959, is based, as we know, on the dipolar character of intereffect of donor and acceptor of energy. As a-consequnce consequence of this, the energy of the intereffect between them is inversely proportional to the third degree of intermolecular distances. Inasmuch as the probability of migration of energy is directly proportional to the square of energy of intereffect, then its effectivity becomes inversely proportional to the sixth degree of intermolecular distances. According to Foerster, the constant of the speed of energy migration may be obtain by the following equation:

$$n_s \cdot \rightarrow A^{\cdot} = \frac{9 \cdot 10^3 \ln 10 k^2}{128 \pi^5 n N \tau_s R^6} \int_{\infty}^{\infty} f_s(\nu) \frac{d\nu}{\nu^4} \qquad (1)$$

where $n_s \cdot A^{\cdot}$ is the number of intermolecular transfers per second; ν is wave number; $\varepsilon_A(\nu)$ is molecular coefficient of acceptor extinction; $f_s(\nu)$ is distribution of energy in the donor fluorescence spectrum;

N is Avogadro number; tau_s is life time of the stimulated state of the donor; n - coefficient os refraction in solvent; R - the average distance between molecules of the donor and acceptor; k is the orientation factor, equal to about 2/3.

If we introduce the concept R_0, i.e. critical distance, at which probability of energy migration and spontaneous deactivation (of fluorescence) become equal, then

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$$N_s \$ \quad A \cdot = \frac{1}{tau_s} \quad \frac{R_0}{R} \qquad (2)$$

where tau_s is life time of the donor, tau_s = =quantum output of donor X life time with energy migration.

The numerical value R_0 for each pair donor-acceptor may be found from the equations (1) and (2):

$$R_0^6 = \frac{9 \cdot 10^5 \cdot \ln 10 / k^2 q_2}{128 \pi^5 n^4 N \nu} \int_{-\infty}^{\infty} f_s(\nu) \varepsilon_A(\nu) d\nu \qquad (3)$$

Steele and Karreman, 1957, offered a simple formula for the calculation od the critial radius R_0:

$$R_0 = \sqrt{0,95 \cdot 10^{-33} \frac{tau_j \bar{\nu}}{\nu_0^{-2}}}, \qquad (4)$$

where tau_s life time of the lower singlet stimulation state of the donor; $j\bar{\nu}$ is integral (area) of overlapping of the fluorescence spectrum of the donor energy with the absorption spectrum of the acceptor; $\bar{\nu}_0$ is the average wave number between the fluorescence and absorption maximum of the donor.

The results of calculations of the critical radius of energy migration between the aromatic amin acids have been presented by

Karreman, Steele and Szent-Gyorgyi, 1958, and are reproduced in Table 13.

Table 13.

Critical radii of energy migration in various donor-acceptor pairs of aromatic aminoacids

Donor-acceptor pairs	tau. 10^8	$\tilde{\nu}_0 . 10^3$	$\tilde{j}\nu . 10^{-8}$	R_0 (Å)
Phenylalanine-phenylananine.. . .	1,6	37,1	0,0404	5,6
Phenylalanine-tyrosine	1.1	37,1	4,1	12,0
Tyrosine-tryptophane tyrosine	1.1	37,1	21,1	16,0
Tyrosine-tryptophane	0,91	34,4	0,458	8,3
Tryptophane-tryptophane.	0,91	34,4	13,0	15,0
	0,20	36,6	0,326	6,3

It should be noted that Karreman et al used for their calculations parameters of tryptophane in aqueous solution. In the case of tryptophane redidues of the majority of proteins, the fluorescence spectrum of which is shifted into the long wave side, as compared to the aqueous solutions on the average of 12-15 mmk (350-335 mmk), the critical radius of the intertryptophane energy migration must be considerably greater, because of the increased integral of the overlapping j tau. We performed respective calculations of the critical radius for tryptophane residues in protein and in the film of polyvonyl alcohol (Table 14). On the other hand, value for the critical radius may be found, based on the ratio of the degree of fluorescence polarisation and the intermolecular distances (of concentration). After Weber, 1954, 1960, from the graphic ratio I/P from the molar concentration C we find the value I/P_∞ with infinitely great dilution, and then based on the formula

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$$R_{exp} = \left[\frac{158 \cdot 10^2}{4\ \mathrm{pi} N \left(1/P_\infty + \frac{1}{3} \right)} \right]^{1/6} (2a)^{\frac{1}{2}} \qquad (5)$$

Table 14

Critical radius of energy migration between tryptophane molecules in protein and in polyvinyl alcohol film

Medium	tau. 10^{-8}	$v_0 . 10^3$	jv. 10^{-8}	R_0 (Å)
Polyvinyl alcohol (max. fl. 320 mmk.)	0.42	33,3	16,0	20,5
Tryptophane residues in hemotrypsinogen (max. fl331 mmk.)	0,16	32,8	10	16,0
Tryptophane residues in pepsin (max. fl. 342 mmk.)	0.45	32,2	4.0	17.0

where N is Avogadro number, a - averafe radius of the molecule, S - the slant of the curve; thus the critical radius is determined.

We propose a simpler graphic method of polarizational determination of the critical radius, still giving satisfactory results, concurring with the results of Weber's method. Inasmuc as at the point R_0 the probability of emission and migration are equal, then practically one-half of the quanta is radiated with the primary stimulation, the other half of the quanta is radiated after the energy migration. This statement is based on the fact that (as we shall show below) the quantum outputs of tryptophane molecule fluorescence at the primary stimulation and stimulation at the expense of the energy migration are equal.

Assuming that this second luminescence is completely depolarized, it is easy to become convined on the law of addition of polarization

$$P = \frac{\sum_i P_i I_i}{I_i} = \frac{0.5\, I_{P_0}}{0.51+0.51} + \frac{0.51 \cdot 0}{0.51+0.51} = \frac{P_0}{2},$$

that with the equal intermolecular distances R_0 polarization of the fluorescence in the system must become twice smaller than the maximum, observed in the

greatly diluted solutions without energy migration. The concentration of the substances, and therefore also R_0 are found easily graphically on the curve of the ratio $p = f(c)$. Comparing the values R_0, found in polarization measurements and calculated theoretically, we see that they are quite close to each other. Thus, from Weber's calculation 1961, for indol and in propylene glycol at -70°C, the experimental 17 Å the theoretical 23 Å (apparently, somewhat elevated), for tryptophane in polyvinyl alcohol the theoretical 20,5 Å, the experimental 21,5 Å (Konev, 1964). The closeness of the theoretical and experimental values proves the resonance mechanism of the energy migration between the molecules.

As for tryptophane in protein, its residues in the average statistical distribution are separated, one from the other as a rule, by the distance 20 Å, i.e. a distance, quite close to the critical radius. The same coincidence of the theoretical and experimental values R_0 is found for the tyrosine (phenol): R_0= 17 A - experimental rate, and 11Å - theoretical rate (Weber, 1961).

Very important for the intermolecular energy dynamics of the protein macromolecule is the problem of the quantum output in the process of intertryptophane energy migration. Is there any loss of energy inthe very process of transfer of energy at the expense of additional expenditure into heat or does the process occur with 100% quantum output? In other words, is there concentrational extinction of tryptophane fluorescence taking place?

The phenomenon of concentrational extinction of fluorescence is well known in the example of staining agents. There exist two contrary points of view as to the cases of concentrational extinction.

The first of these, expressed by V.L. Levshin back in 1927, consists in the thought that energy migration, by itself, is not accompanied by loss of energy of electron stimulation, but the effect of extinction is conditioned, first of all, by the inactive absorption of associates, occurring in high concentrations, and, in the second place, by the energy migration to them from the monomers. Another point of view was expressed by Galanin, 1960, on extinction at the moment of migration from the monomer to monomer; this is less thoroughly documented.

The concentrational extinction of fluorescence of aquaeous solutions of tryptophane has been described by Vladimirov in 1957 and confirmed by Konev, Katibnikov and Petrova in 1961 for the water-glycerine solution. however, contrary to the staining dyes, in the concentrated aquwous solutions of tryptophane there is no formation of dimers or associates. This is supported by the character of the ratio of concentrational extinction to the wave lengths of the stimulating light. Vladimirov established, 1957, for three lengths of waves of stimulating light, and Konev, Katibnikov and Lyskova, 1964, for the entire spectral region 220-300 mmk., the identity of the forms of the curves of concentrational extinction (Fig. 43). This shows the absence of the associates in the tested solutions, since in the latter case for those wave lengths which would coincide with the maximum of absorption of the associate, there would have been observed more intensive extinction of fluorescence. Whereas, identical extinction for the entire spectral region 220-300 mmk indicates the unchanged spectrum of absorption of the whole fund of molecules in the solution in proportion to the increased concentration. Discarding the possi-

bility of the appearance of concentrational extinction as a result of formation of dimers or other associates, we had to assume that extinction is conditioned by the processes of energy migration from monomer to monomer. However, this hypothesis, again, proved to be incorrect. It was clarified (Konev, Katibnikoff, Lyskova, 1964) that in proportion to the increase in viscosity of the solvent the concentrational extinction gradually decreases and with high rates of viscosity (1000 cn and over) it disappears completely (Fig. 44). In the case of solid (hard) films of polyvinyl alcohol, the concentrational extinction was not observed even up to the concentration of 1 M/l, at which tryptophane fluorescence was compptely depolarized as result of energy migration (Fig. 14,a, curve 1). Therefore, contrary to the dyestuffs, in which extinction frequently has a statistical character and does not depend upon viscosity (Levshin, Krotova, 1960), tryptophane fluorescence extinction has a clear-cut kinetic nature and is not associated with the course of energy migration processes. Only at the moment of collison of the stimulated molecule of tryptophane with the non stimulated/are created conditions for effective extinction. The most important circumstance, for us at the given moment, consists in that energy migration does not produce decrease in quantum output of fluorescence. This makes it possible to draw the conclusion that the quantum output of the migration process between the tryptophane molecules is equal to 1.0. Moreover, stimulation of the molecules, at the expense of the energy migration, is about five times more effective than their stimulation by the quanta of light. Inasmuch as the quantum output of tryptophane fluorescence is equal to 0.2, this means that of the five quanta

of the stimulating light, absorbed by the molecule, only one leads to the creation of fluorescence in the molecule. But in the case of the inter-tryptophane migration of energy, each and every picked up quantum of energy results in the appearance of flurescencent state. Therefore, the migration path of molecules stimulation is much more effective that the radiation path (by way of direct stimulation with the quanta).

Fig. 43. Concentrational extinction of aqueous solutions of tryptophane (pH 10) with stimulation of fluorescence with light of various length waves: 1 - 220; 2 - 248; 3 - 254; 4 - 265; 5 - 280; 6 - 290; 7 - 302 mmk. Room temperature.

Fig. 44. Concentrational extinction of fluorescence in tryptophane solutions:
1 - in aqueous solution (pH 10): 2 - in water-glycerine mixture 1:1 (pH 10); 3 - in dehydrated glycerine (dry NaOH was added to attain with induction paper pH 10). Room temperature.

Taking into consideration the above statements, it may be assumed that a/characterstic of intertryptophane migration of energy in proteins, occurring according to the mechanism of inductive resonance along the singlet levels ^1L_a of tryptophae molecules, is the 100% quantum output of energy transfer.

However, for the evaluation of the specific role of energy migration in the energetics of protein macromolecule, particular importance, along with the quantum output of energy migration, belongs to its effectivity, i. .e the percentage of "delocalized" tryptopnae states, chnaing position of their original appearance of fluorescence stimu

mulated states, with reference to the total amount of fluorescent states, appearing in a unit of time. Precisely the probability of transfer of the fluorescent state, appearing as result of absorption of quanta of light in the macromolecule of protein, which may be transferred to another tryptophane residue into another portion of protein molecule, - this is what determined the rol of energy migration in photochemistry and photbiology.

Assuming, along with Galanin, that fluorescence, even after single act of transfer of energy, is practically completely depolarized, we shall write the formerly given formula in this form: $\frac{P_o - P}{P_o} = \frac{\sum I_k - I_o}{I_k} = \eta$, where η determines the effectivity of the energy migration.

Inasmuch as the constant of the energy migration speed, and therefore also its effectivity, is proportional to $E = \frac{I}{(R_o/R)^6 + I}$, then the energy migration effectivity must greatly depend upon the distances between the tryptophane residues, i.e. upon the inter-protein topography of this aminoacid. From this formula, it follows for instance, that with R_o for the typtophane residues in protein beling equal to 16Å, devreasing the distance only by 3Å would be accompanied by increased effectivity of energy migration from 50 to 77%, while decreasing the distance to 10Å would lead to increased effectivity of energy migration up to 95%. As a result of this, we may a priori expect sonsiderable variations in the energy migration effectivity in various proteins. Actually, evaluation, done with the above quoted formula, gives the following values of energy migration effectivity (Table 15).

Table 15

Effectivity of Intertryptophane Energy Migration in Various Proteins

Protein	Degree of Polarization P_{278}^{335}	Effectivity of energy migration %
Human serum albumin	14,0	0.0 [1]
Pepsin	11,8	55
Edestin	8,6	67
Gamma-globulin of pumpkin seed	15,5	40
Hemotrypsinogen	9,5	64
Trypsin	12,1	53
hemotrypsin	12,0	54
Globin	12,2	53
Hemoglobin	21	19
Silk fibroin	6,0	77
Wool keratin	5,0	81
Cytochrome c	7,0	73

[1]) Depolarization of fluorescence was produced by tyrosine-tryotpphane energy migration.

In the case of wool keratine, the degree of fluorescence polarization proved to be closely dependent upon the conformation of the protein molecule, and the type of configuration of its polypeptid chain (Konev, Katibnikov, 1961). Transfer of the alpha-configuraion of the peptid chain into the beta-configuration by means of extraction of wool fibers with water steam twice is accompanied by increase in the degree of fluorescence polarization from 4 to 20-25% (Fug. 18 and 33). Data of roentgen-structural analysis show that the transfer (conversion) from alpha into beta-configuration is reversible.

In complete agreement with this, after the removal of the tightening effort, the degree of fluorescence polarization gradually decreases to the initial level (4-5%). Therefore, in this case, the polarization fluorescence reflects the structural reconstructions which take place in the protein molecules. At the same time, changes

in the degree of polarization of fluorescence are essentially conditioned by the orientation, rather than the migration effects. In favor of the orientation nature of the appearance of polarization luminescence of wool keratin speak a number of facts and, first of all, the depolarization of fluorescence of wool, extracted with steam, in the case, when its long axis is oriented perpendicular to the vector of stimulating light or the fibers are oriented chaotically. This directly indicates that in the main portion of tryptophane residues intensive process of energy migration continue and only a portion of the residues, because of their orientation, emanates polarized luminescence.

In the second place, the presence of oriented zones of tryptophane residues follows from the existence of spontaneous fluorescence polarization during its stimulation by non polarized light (+4% and - 4%).

Finally, the effect of orientation is manifested in the possibility og obtaining polarization spectrum of fluorescence after absorption with stimulation of vertically oriented wool, extracted with steam, by horizontally oriented vector of stimulating light (Fig. 33, curve 8). This polarization spectrum, as mentioned above, reflects the spectrum of absorption (transfer) passage of the electron passage of ^1L_b oriented residues of tryptophane and may appear only in the case of orientation of molecular axes with reference to the axis of the wool fibers (Fig. 45).

Fig. 45. Diagram of disposition of tryptophane residues with reference to keratin fibrilles.

The main biologically important result of energy migration is the

shifting of the electron stimulated states in space, as though increasing the transverse section of effect (capturing) of the ultra violet quanta. Another not insignificant role of the primary photophysical processes, following the act of absorption of energy, may consist of separation, in time, of the moment of absorption of the quanta and the performance of its chemical effect. Such an increase of the period of time of the effect of the quanta, along with the enlargement of the spatial sphere of their action, may prove to be, in the photo- and radio-biology a very essential moment. In this connection, there arises a question, closely associated with energy migration, as to possible prolonged preservation of energy of electron stimulation in the protein systems. Inasmuch as vital processes occur at plus temperatures, we will be first of all interested in the problem of possible prolonged preservation of energy of electron stimulation, precisely at comparatively high temperatures, i.e. at room temperature. At the same time, we shall attempt to elucidate the role of tryptophane in the mechanism of energy pre# servation.

The earliest indications as to the possible after-luminescence of dry powders of proteins at room temperature are found in the work of Vladimirov and Litvin, 1960, who registered in the interval of 1-5 sec. after radiation with ultra violet light, in arachine powder a ~~short~~ after luminescence with accentuated maximums 435 and 455-460 mmk. At the same time, they succeeded in registering semiluminescence of proteins in solution.

Konev and Kalibnikov studied the kinetics of after-luminescence of protein in solution and in transparent films, obtained by drying

at the temperature of +40 - 50°C, In the concentrated aqueous solutions of proteions (1961, 1961). Fig. 46 illustrates curves of after-luminescence of films of various proteins after preliminary radiation with ultraviolet slight (250-40 mmk.) during 15 sec. A study of these curves shows that extinction of the intensity of post-luminescence in the protein films occurs in two phases: a quick, almost exponential decrease in intensity during the first 30 sec. is then changed to a slowly extinghuishing luminescence, lasting many minutes. The protein which does not contain aromatic aminoacids, clupeine, as found by measuring, possesses afterluminescence by 1,5 - 2 order of degree less intense than the after (post)-luminescence of the tryptophane-containing proteins. This fact indicates causative relationship between the tryptophane contents in tprotein and the property of postluminescaece at room temperature. That this portion of the post radiation belongs to the tryptophaner molecules may be clearly seen from the form of the spectra of stimulation of the first phase of after-luminescence, in which there are marked both bands of absorption of tryptophane residues in protein - at 280 mmk and at 225 mmk, separated by the minimum at 240 mmk (Fig. 47). The after-luminescence spectrum as a whole coincides with the phosphorescence spectrum of proteins at low temperature, but it is somewhat shifted into the long wave side, as compared to it, because the maximum of luminescence is shofted from thevibratory band 438 mmk to the arm (shoulder) 470-480 mmk. Gradual redistribution of the intensity of macimums, expressed in the increase of intensities of the long wave component, may be seen in thking spectra in proportion to raising the temperature from -30 -50° C. up to the room temperature. In a number of proteins, for instance milk casein

granules, the structure of the phosphorescence spectrum is preserved even at room temperature. Taking into consideration the tryptophane nature of the after-luminescence of proteins at room temperature, it remained to explain with what centers may be associated, although only 1,5 - 2 times weaker, but still quite distinct after (post) radiation (luminescence of clupeine protein which does not contain even one single aromatic aminoacid.

Fig. 46. a - kinetic curves of decrease in intensity of afterluminescence of various proteins in film after radiation with a lamp SVD-20 A at a distance og 10 cm. during 15 sec.; 1 - wool keratin; 2 - amylase in film; 3 - human serum albumine in film; 4 - silk fibroin; 5 -barley roots; 6 - clupeine; 7 - pepsin; b - aqueous solutions: 1 - gamma-globulin of human serum albumine; 2 - human serum albumine; 3 - clupeine; 4 - asparagin (0,1% aqueous solution).

Fig 47 Spectra f sti umatinn f the first stage f p st-luminescence f pepsin films (1) and amylase (20 at room temperature.

Fig 48 Spectral distribution of after-luminescence of films of Na-carboxymethyl cellulose (1) and clupeine (2) at room temperature.

From Fig. 48 one can see that the stimulation spectrum of post-luminescence of clupeine sharply differs from the corresponding spectra of tryptophane-containing proteins. In the clupeine spectrum, there is observed only one band with maximum at 270 mmk. Light with waves longer than 300 mmk and shorter than 225 mmk stilumated no after-luminescnce. Likewise, it is striking that the spectrum of after luminescence stimulation in clupeine does not at all coincide with

the spectrum of its absorption which has no absorption in the region of 270 mmk. It is characteristical that the water-soluble Na-carboxyθ-methyl cellulose in film also gives the same maximum of the same intensity in the stimulation spectrum. P ssibly, in both cases the carboxyl groups are the chroomophores; their maximum of weak absorption belongs in the region of 270-280 mmk. (Bredereck, Hoechele, Huber, 1953; Schur, 1957).

Therefore, the nature of the centers, responsible for the after-(post)-luminescence of clupeine is different than in the tryptophane-containing proteins and accordingly the intensity of the after-luminescence is about 100 lower than in the remaining proteins.

In this manner, a peculiarity of the energetics of the protein molecule is the potential creation in them, even at room temperature, of quite noticeable stationary concentrations of molecules in triplet electronstimulated state. It is characteristical that the triplet states of the tryptophane residues in proteins may appear not alone at the expense of the optical, but also at the expense of the chemical stimulation. Of particular interest is the second kinetic stage of after-luminescence of proteins. The most intense after-luminescence at this stage is observed in proteins, rich in sulfur-containing aminoacids, - wool keratin and pepsin. The f different mechanism of luminescence in the first and second stages may be expressed not alone in kinetics, but also in obvious thermo-active mature of the second stage. Elevation of temperature up to 75°C is accompanied by sharp (ten-times) decrease is luminescence of wool keratine in the first stage and, on the contrary, its increase in the second stage (Fig. 49.). Increase intensity of after-lumines-

cence with the rise in temperature, for the second phase, follows the exponential law (Fig. 50). The energy of the activation of

ccal? this process for keratine of wool amounted to 21,5 kkal/mol. It is remarkable that the same rate of energy activation was found by Riehl, 1956 for the electoconductivity of water-containing proteins. The activational character of after-luminescence is also found in silk fibroinm amy ase films, pepsin, albumine and gamma gl buine of human/blood serum.

Fig. 49. a - effect of temperature on the kinetics of post luminnescence of wool after preliminary radiation during 15 sec. with ultra violet rays (250-380 mmk.): 1 - wool at 75°; 2 - humidified wool at 20°; 3 - wool at 20°; 4 - humidified wool at 75°' 5 - influence of oxygen on yhe kinetics of post luminescence of wool after its preliminary radiation under the same conditi ns; 1 - humidified wool under vacuum; 2 - wo l under vacuum; 3 - wool at atm rpheric pressure; 4 - wool at atmospheric pressure, humidified.

Fig 50 Dependence (rati) of intensity of post luminescence upon the temperature.

The presence of two mechanisms of post luminescence of proteins at the room temperature is also manifested in the character of influence of outside effect on each stage of after luminescence f proteins. In the dry specimens of wool keratin, oxygen decreases the intensity of luminescence in the first stage, not essentially affecting the second stage In the absence of oxygen, humdification of the specimen results in increased luminescence in the first stage

and, on the contrary, in a slight decrease in the second stage (Fig. 49). The activationcharacter of the second stage of after-luminescence of protein directly indicates the apperance of the quanta of light as the result of recomination processes. There arises the natural question what process is activated by added thermal energy: the physical process of repulsion of the electron from the trap into the zone of protein conductivity or else the chemical process of recombination of the products of the primary photochemical reaction, for instance, recombination of ion-radical of tryptophane with the radical pf the microsurroudnings, arising as the result of the capture of the photoelectron by the "solvent". In other words, are we dealing with the mechanism of crystallophosphores or the mechanism of hemiluminescence? First of all, the data of paramagnetic resonance show formation of a signal in protein, after the effect of ionizing radiation and ultraviolet, this signal being conditioned by the non evaporated electrons (Blumenfeld, 1957; Eidus, Kaiushin, 1960; Eidus, 1960; Patten, Gordy, 1960 and others). At the same time, of greatest interest is the data of Sapeshnike Sapeshinskii, Silaev and Emanuel, 1963, who observed good parallelism in the weakening of the signal EPR and the intensity of post luminescence in the protein systems radiated with the ultraviolet light. In the light of these findings, the participation of certain elements with non evaporated electrons in the mechanism of after luminescence appears to be incontestable. However, the carriers of the EPR signal could be both the products of the photochemical reaction - radicals, and the electrons, caught on the trap near the zone of conductivity. In favor of the semiconductor mechanism we might cite, for instance, the fact of coincidence of energy activation

EPR? abbreviation not identified

of energy activation of electroconductivity of proteins and after luminescence. However, the presence of the second phase of after luminescence, both in the native and in the denaturated proteins still induces us to prefer the hemoluminescent mechanism of the appearance of post luminescence. Such a mechanism of the origin of the second stage of post luminescence becomes the more probable, since we have registered (Konev, Katibnikov, 1961) for gamma globulin in aqueous solution, and then Vladimirov, Litvin and Li Man Si, 1962, for aminoacids proper, a clearly marked hemoluminescence, depending upon the oxygen pressure. Hence it is possible to explain also the pronounced dependence of the intensity of the second stage of after luminescence upon the viscosity, stiffness in the organization of the albumine substratum Precisely the viscosity of the medium is, apparently, the fact which prevents instantaneous reunion of the built up free radicals Further studies of these peculiarities in this phase of after luminescence permits us to accept the following diagram for the processes of its origin:

1) trypt. + hv $\rightarrow$ trypt.^{s},
2) trypt.^{s}, $\rightarrow$ trypt.^{T}
3) trypt.^{T} + A $\rightarrow$ trypt.$^{R+}_{T}$ + A - $\bar{e}$
4) trypt.$^{R+}_{T}$ + $\bar{e}$ - A $\rightarrow$ trypt, + A
5) trypt. $\rightarrow$ typt. + hv
phosph.

The role of tryptophane as acceptor of quanta of aultra violet light is confirmed by the coincidence of the spectra of stimulation of the first and second phases of after luminescence (experiments with light filtration) The first phase, in its turb, with monochromatic stimulation has a tryptophane stimulation spectrum.

The appearance of the quanta of light in the second phase, as a result (consequence) of triplet-singlet transfer (passage) in the

recombined tryptophane molecule becomes clear from the study of the spectral composition of this luminescence. Complete absence of luminescence with maximum at 350 mmk., corresponding to singlet-singlet transfer and, conversely, the nearness of the spectral composition of the second phase of after luminescence of tryptophane phosphorescence (although somewhat shifted into the long wave side) confirm the above diagram. The proposed diagram of the origin of prolonged luminescence of tryptophane residues, generally speaking, is not at all unusual. Prolonged luminescence of a number of organic substances in glasses at room temperature, associated with the photo-(uncoupled)
lectron dissociation and its delay in the non paired state in the
1944
solvent, has been observed, as we know, by Lewis, Kasha, 1947, Linschitz et al, 1954 and a number of other authors.

A certain specificity of the above diagram consists, however, in that in this case the process occurs not through the singlet, but through the triplet electron-stimulated states of tryptophane. Essentially, we are dealing in this case with a peculiar process of hemophosphorescence, the possibility of which has been questioned by many authors, including such well known research sicnetist as Rid, 1960.

Therefore, in the most general way, the chain of events leading up to after (post) luminescence, may be described in the shortest way as follows:

1. Absorption of energy by the tryptophane molecules with their passage (transfer, change, conversion) into the triplet state and dissociation of photoelectrons.

2. Capture and preservation (storage) of photoelectrons by the supramolecular enriomment with formation of radicAls.

3 Reverse reaction, activated by the thermal (heat) quanta, recombination of tryptophane residues with the dissociated electrons andluminescnce from the triplet levels of tryptophane.

CHAPTER IV

Luminescence of Nucleic Acids and Energy Migration In them

The history of the study of luminescence in nucleic acids greatly resembles the history of the study of protein luminescence. In the 30's and 40's there appeared a considerable number of works, describing the visible (violet, blue, purple, green) fluorescence, both of the free bases and their derivatives, and the nucleic acids proper, stimulated with light 365 mmk (Neyroth and Lofbourow, 1931; Euler, 1035; Lofbur w, C ok and Stimson, 1938; Stimson, Reater, 1941 and others). For instance, in the latter work there was visually registered luminescence of β w/ 20 derivatibe nitrous nitrogen bases, including uracil, 2,6-dichlorpyramidine, cyt sine, is cytosine, guanine, isoguanine, adenosinephosphoric acid, as well as nucleic acids from yeast and calf thymus, in solid state, in NaOH, NH_4OH, H_2SO_4. Weakening of luminescence intensity in proportion to purification, the possibility of stimulating it outside of the absorption band, the least intensity of luminescence in guanine in the acid medium, i.e. precisely where it possesses the greatest fluorescence as compared to other bases, - all these factors leave no doubt that the above mentioned authors were actually dealing with luminescence of some admixtures, ~~intea-~~ instead of the basic substance.

Further, more perfected, spectrofluorometrical studies showed that in the neutral aqueous solutions, at room temperature, neither purine, nor pyrimidine bases have the capacity to luminescnce either

in the ultraviolet or in the visible regions of the spectrum.

In solutions at room temperature, there has been described only one luorescence of purine bases - guanine and adenine - in strongly acid medium - in 0.1 N HCl (Duggan,Bowman, 1957; Barskii, 1959; Agroskin, Korolev and others, 1961). At the same time, on the curve of the acid titration of fluorescence intensity of guanine I=f(pH) which is s-shaped, the point of half-extinction is near to the point f transfer of half of guanin molecules into the tautomeral form; the semi point of half-extinction is near the point of transfer of half of guanine molecules int the tautomeral form (Barenboim, 1963). In the acid medium, the fluorescence maximum of guanine is at 355 mmk., that of adenine at 365 mmk. The stimultaione fluorescence spectra are close, although not identical, to the spectra of their absorpti n (Agroskin, K r lev, and other, 1961).

In the 7 n hydrochloric acid, guanine fluorescence has maximum 365 mmk (Duggan, Brody, Udenfriend, 1957; Barskii, 1959), while with pH 1 maximum is shifted into the short wave side to 352 mmk (Barskii, 1959).

The phosphorescence of adenylic compounds at the temperature of 194°K has been described by Steel and Szent Gyorgyi, 1957. The kinetics of extinction was satisfactorily described by the equation of the first order, the constant of speed of extinction did not depend upon the temperature; this led the authors toassociating of the after luminescence of the adenylic compounds with the triplet state of the molecules. Contrary to Steel and Szent Gyiorgyi, who were dealing with snow-like frozen specimens, Bersohn and Isenberg, 1964, worked with water-glycerine transparent glass at the temperature of 77°K and

registered with a photoscope with permissible time 10^{-3} sec. a well structured adenine phsophorescence spectrum (desoxyadenosine monoph sphate) with maximums at 390, 415, 445 and 60 mmk. and non structured spectrum of guoanine (desoxyguanozinem n phsophate) with maximum at 420 mmk. The derivatives of the pyrimidine bases (radicals?) under the same conditi ns sh wed bo registered phosphorescence. It may be assumed that freezing solidly fixates the ring of the bases and interferes with the extremely effective n n radiated path f dissipation f energy. In a similar manner, not only freezing, but also other methods of fastening the skeleton of the molecule result in the appearance of the luminescence property. Fluorescence arises with adsorption of the bases on the chromatographic paper (Barskii, k959) and with freezing of dry powders of bases and their derivatives down to the temperature of liquid oxygen ~~90°K~~ -- 90°K. In the latter case, there appears distinct luminescence, although with a very low output (apparently, of the order of 1%), with washed-out maximums, situated within the limits of 320-400 mmk. The position of the maximums is represented in Tabel 16, borrowed from the work of Agroskin, Korolev nd others, 1960. Apparently, the solid, or more exactly powder-like aggregate condition of the specimen itself it not at all required for the appearance of fluorescence. Bersohn and Isenberg, 1964, ~~registrated~~ registered thymine fluorescence and cytosine fluorescence in water-glycerine candy at the temperature of 77°K with the same maximum at 340-350 mmk; this somewhat varies from the values of the Soviet authors, due to inexact corrections for spectral sensitivity.

The earliest attempts to find ultra violet luminescence in nucleic acids were unsuccessful, essentially because of ~~xxxxx~~ little sensi-

tivity of the apparatus (Shore and Pardee, 1956).

Later there was demonstrated the capacity of the nuceinic acids to fluoresce and phosphoresce: Konev, 1957, described fluorescence

Table 16

Position of the maximums of luminescence in powders of nuceinic acids and their chromophores at t=~~90~~ 90 K

Substance	Maximum f spectrum mmk		Substance	Maximum f spectrum mmk	
	lambda stimul. 260-280 mmk	lambda stimul 296-313 mmk		lambda stim 260-280 mmk	lambda stim. 296-313 mmk
Adenine	355	355	Uracil	355	430
Adenosine			Uridine-5-monophosphate	320	430
Adenosine-5-monophosphate	360	350	Uridine-5-triphosphate	330	340
Guanine	370	370	thymine	310	395
Guanosine	325	355	Thymidine-5-monophosphate	335	375
Guanosine-5-monophosphate	370	360	RNK ribonucleic acid?	350-360	380-400
Cytosine	400	400	DNK deoxyribonucleic acid	340-360	350-380
Cytidine-5-monophosphate	330	340	various polyphosphates	320-350	320-350

in nucleohistone of the thymus of calf, and Douzou, Franky Francq, Hanss, Ptak, 1961, - fluorescence and phosphorescence in the same object.

Isenberg and others, 1964, for the deoxyribonucleic acid of calf thymus, salmon sperm and 2T-phago- obtained stimulation phosphorescence spectra with maximum at 295-300 mmk; these did not coincide with basic long wave maximum of absorption. Similar stimulation spectra were obtained by the French authors for the deoxyribonucleic acid (300 mmk), polyuridylic acid (311 mmk) and polyadenylic acid (270 mmk). Based on the deciphering of the nature of the electron spde spectra of absorption of nitric bases, done by Kasha, 1961, ht he

Feench authors assumed that fluorescence of the nuceinic acids is conditioned solely by the n - pi passages (transfers).

From the data of the same authors, the fluorescence spectrum of the deoxyribonucleic acid has its most intensive maximum at 355 mmk and two weaker maximums at 325 and 390 mmk. It should be stressed, however, that the quantum output of fluorescence in deoxyribonucleic acid in aqueous solution is quite low. A marked luminescence of the nucleic acids in aqueous solutions at room temperature is observed in the strongly acid medium (Agroskin, Korolev and others, 1961; Barencoim, 1963; Doudou et al, 1962). At the same time, maximum of fluorescence of DN acid is at 355 mmk and coincides with the maximum of fluorescence of guanine. The spectra of action likewise coincide with the guanine absorption. In the low polymer ribonucleic acid, highly molecular nucleic acid from yeast, low polymer DN acid and highly molecular DN acid from calf thymus gland, the stimulation spectrum if repesented as varying in maximum in shape and height at 250 And 275-280 mmk. The lack of concordance of this spectrum with the absorption spectrum of RN and DN acids, as a whole, shows not alone the predominAnce of guanine luminescence, but likewise the absence of sufficiently effective migration into it of the remaining bases. The exclusive role of guanine is confirmed also by the fact that the nucleinic acid, deprived of purines - apyrinic acid - does not have luminescence, whereas removal of pyrimidines has little effect on the spectral luminescence properties (Agroskin, Korolev and other, 1961). According to the findings of Barenboim, 1963, DN acid is luminescent in the solution, only starting with pH 4,2, and the s-shaped curve of titration of fluorescence intensity coincudes with the acid titra-

tion of guanine; this presents another reason in favor of hthe conditioning of DN acid fluorescence by the acid tautomeric (tautomeral) form of guanine. But, even in these conditions, its quantum output is very low - not exceeding 0,3-0,5% (Konev, 1964).

The most interesting peculiar feature of nucleinic acid luminescence in aqueous solutions at room temperat re is, quite likely, that fact that at change-over to weakly alkaline reactions in DN acid (phosphate buffer pH 7,2) (Agroskin, Korolev, and others, 1961), and histone?
in the case of nucleohystone, both in water and hydrophosphate solvent (Konev, 1957), there appears again quite intensive fluorescence, but this time with the main maximum of stimulation at 290-300 mmk. There are some reasons for associating this luminsecence with n # pi transfers (passages) inthe macromolecule ofDN acid. In the columns, oriented plane to plane of the bases, the passages n - pi are directed parallel to each other along the course of the double spiral, and therefore there is possible strengthening of this passage at the expense of the intereffect. The distinguishing property of the n - pi passages is their disappearance with acidification (oxidation) of the medium; then it is found that the acidDN acid has a maximum in the stimulation spectrum at 250 mmk. In the weakly alkaline medium, on the other hand, there may take place intereffect of the passages, not alone in guanine, but in all the remaining bases, and in this case, the band will be quite sensitive to all sort of disturbarces in the arrangement of the secondary and tertiary structure.

Other peculiarities of luminescence are manifested in nucleinic acids in powder. Under these conditions, now all of nitric bases participate in the formation of fluorescence spectrum of DN acid (Ag-

roskin, Korolev, Kulaev, Meisel', Pomoshchnikova, 1960; Barenboim, 1963).

Considerably more marked, as compared to fluorescence, is the phosphorescence property of the nucelinic acids. The phosphorescence of water-glucose solutions of nucelinic acids at the temperature of liquid nitrogen has been observed back in 1957 by Steel and Szent Gyorgyi. While Douzou et al, 1961, gave for the snow-like water aqueous DN acid at the temperature of 77°K a non structural band of phospho# rescence w th maximum at 475 mmk, Bersohn And Isenberg, 1964, working with water-glycerine glass of DN acid, registered separate structural elements in phosphorescence spectrum, with the use of phosphoscope resolution? with permissible time of 10^{-3} sec. It is characteristical that the phosphorescence spectrum was then found to be formed as an addition from the adenine phosphorescence spectra - the maximums in the free desoxyadenosinemonophosphate at 390, 415, 445 and 460 mmk and in guanine - desoxyguanosine-monophsophate has a wide maximum at 420 mmk. From the data of Bersohn and Isenberg, 1964, pyrimidines - cytosine and thymine - have no phosphorescence at the temperature of 77°K, but they do fluurescenc with maximum at 340-350 mmk.

However, in this case, the absence of phosphorescence, apparently, does not at all mean the abseence of tri let electron-stimulated states. As a matter of fact, pyrimidines, in triplet state, have a low pi-electron density between the carbon atoms 5 - 6 (Mantione and Pullman, 1964); this is equvalent to a break of the bond. This may result in 100% loss of stimulation energy in the course of the photochemical reaction of hydration or simply in the course of effective variations of 5-6 bond. Theoretical considerations and some characteristical

peculiarities of DN acid phosphorescence led Bersohn and Isenberg to propose an extermely attractive hypothesis on the exciton (? English?) triplet mechanism of the energy migration in the electron stimulated state along the bispiral of the macromolecule. One of the main experimental bases fo these conceptions were experiments on the qualitative studies of phosphorescence extinction with the paramagnetic ions. It was found that only one paramagnetic ion is capable of extinguishing phosphorescence of many bases - it seems to sort of suck away the energy noy only from that base to which is it directly "attachex", but also from those bases which are not in contact with it and are removed from it in space. At the same time, there was observed no fluorescence extinction as a parallel to phosphorescence (extinction); this means energetical generalizztion not alone/the of sigglet but also the triplet levels. From these authors calculations, the time of localization of exciton at individual base is lesser than 10^{-9}sec. At the same time, the representation (presence) in the phosphorescence spectra of DN acids, both of adenine, and guoanine, dependence of the extinction time upon the wave length, exclude the possibility of transfer of energy between adenine and guanine, leaving the possibility of the appearance of exciton pnly between homogeneous molecules. Concepts on the triplet nature of delocalization of the electron-stimulated state in the DN acids is considerably supported by the experiments of Isenberg, Leslie and others (1964). These authors, while studying the complexes of acrydinic dyes with DN acids, found in them three forms of luminescence: DN acid phosphorescence, phosphorescence of the staining agent, and

retarded (delayed) fluorescence of the dye. Precisely the latter luminescence is of the greatest interest, inasmuch as it is not stimulated by the direct appearance of the quanta in the molecule itself of the dye, but is stimulated only indirectly, by the quanta, abosrbed by the nitric bases of the DN acid. The intensity of the retarded luminescence, with increased intensity of stimulating light, growth linearly with the intensity of phosphorescence of DN acid, i.e. the concentration of the triplets. The fact that during the life time of the retarded luminescence is shortened from $5 \cdot 10^{-2}$ to 10^{-1} sec. in proportion to the increased number of molecules in the dye, belonging to nitric base, led these authors to the conclusion that here at first there occurs a triplet-triplet migration of energy between the nitrious bases until "collision" with the nearest molecule of the dye, and then energy migration into it (triplet-singlet mechanism). I^N the estimation of the authors, the time of migration between the neighboring molecules of guoanine along the spiral DN acid is 10^{-4} - 10^{-1} sec. Rahn, Longworth et al, 1964, also admit delocalization of the triplets in the synthetic polynuceotids. On the other hand, delocalization of the electron-stimulated states in the nuceic acids is possible, apparently, also with the first singlet state of purines (Weil, Calvin, 1963; Gagloev, Vladimirov, 1964).

CHAPTER V

Biopolymer Luminescence in the Cell

The priority of the investigation of the primary luminescence, proper to the cells, situated in the ultra violet region of the spectrum and conditioned by protein, as proved at this time, belongs

completely to the Soviet scientists.

In 1958, E. M Brumberg used the ultra violet luminescence microscoope MUF, which he developed for the study of the primary luminescence of biological objects. The very first investigation showed that the cells of microbic, plant or animal origin possess an intensive ultraviolet luminescnce, stimulated by the short wave portion of the ultra violet spectrum (250-280 mmk.). Simultaneously and independent from the studies by Brumber and Meisel et al, Vladimirov, 1957, using a different method of macroscopical examination of the stimulation spectrum of fluorescence of yeast cell suspencion, came to the conclusion that the yeast cells give ultra violet fluorescence which passes through light filter UFS-3 and has a stimulation spectrum with maximum of 280 mmk. This circumstance he interpreted as a proof that the luminescence center in the yeast cells is protein. We maypresent two questions, around which are centered all the research studies in the field of primary ultra violet luminescence of cells and tissues. In the first place, this question of the nature of luimenscence centers in the cell, of the nature of those molecules, in which the electron-stimulated states are responsible for the luminescence center of the cell, on the one hand, and those centers which play the role of sensibilizators of this luminescence, on the other hand.

In the second place, the question of the prime importance in biology is that of the interrelationship of the luminescence cell and its physiological state, that of the correlation between the functional or pathological state of the cell and its spectral and luminescence characteristics.

Let us briefly review the thus far accumulated sum total of experimental data and conclusions derived therefrom, for each one of these questions separately.

On the Nature of the Centers, Responsible for the Primary Ultra Violet Luminescence of the Cell

The problem of the primary luminescence of the cell is quite complex. Brumberg, BArskii and Shudel", 1960, in their earliest works, believed that the fluorescence of cell nuclei is conditioned by the nucleinic acids, whilte the cytoplasm, by the ribonucleic acids and proteins. However, subsequent experiments showed that gradual removal from the cells of the free nucleotids, ribonucleic and desoxyribonucleic acid, is accompanied by a sudden sharp drop in the intensity of luminescence (Brumberg, Barskii, Shudel', 1960); this led them to the conclusion about the nuecleotid nature of the luminescence. Soon this conclusion met with an apparent contradiction in the fact that in the stimulation fluorescence spectra of the cells of rat bone marrow, and mouse liver and spermatids cells (Agroskin, Barskii, 1961), the maximum appears at 280-290 mmk.; it resembles the maximum in protein stimulation spectra. The conclusion about the protein nature of luminescence was also reached in the experiments of Agroskin and Pomoshchnikova, 1962, who confirmed the maximum 284 mmk. in the stimulation spectra of yeast and a number of microbic cells: Sach. cer. 12, Sach. luwigi, 10, Endomyces magnusci, E. coli, B. Anthrocoides, Micrococcus rubi, B. mycoides, Pseudomonas fluoresc. lignifaciens.

It wuuld seem, these experiments should bring about the conclusion on the monopoly role fo proteins as a center of luminescence in the

were
cells, was it not known that in a number of cases both the nucelinic and nuceoproteid acids have the same long wave maximums of stimulation (Konev, 1957; Agroskin, Korolev, Kulaev, Pomoshchnikova, 1961; Douzou, Francq, Hans and Ptak, 1961; Isenberg and others, 1964).

In more recent works of Brumberg and coblaborators, 1961-64, he frequently returned to the problem of the nature of the primary ultra violet luminescence of the cells. He mentioned two more arguments agAinst the protein nature of cellular luminescence. First of all, there is observed the absence of direct connection between the tryptophane content in the cells and the intensity of the luminescence (Barskii, E. Brumberh and B. Brumberg, 1962; Pil'shchik and Nikolaeva, 1963). In the opinion of Brumberg, Barski, Chernogriadskaia and Shudel'. 1963, against the protein nature of luminescence also was the high sensitivity of this fluoresccence to the changes in the physiological state of the cells which had to "admit the presence of marked dependence of the latter from (upon) the protein struc. (page 150). The picture of the ultra violet luminescence of the cell became still further complicated after the publication of two articles - that of Chernogriadskaia and Shudel', 1962, and that of Brumberg, Barskii, Chernogriadskaia and Shudel', 1963. In the first of these, it was stated that the luminescence in the cells is conditioned by the oxidized form of diphopyridine nucleaotid. This conclusion of the authors was based on an apparent misunderstanding, since the restored diphosphopyridin nucleotid luminesces not in the ultra violet, but in the visible portion of the spectrum with maximum at 465 mmk (Velik, 1961), while the oxidized generally does not dluoresce. In the second work, Brumberg et al draw attention to the fact that

tion to the fact that the prosthetic group of one of the respiratory ferments - ubiquinone or coenzyme Q - does fluorescence in reconstructed form inthe ultra violet portion of the spectrum. At the same time, a rekated compaound to ubiquinone - hydroquinone - has a fluorescence spectrum with maximum at 325 mmk. and stimulation spectrum with maximum at 290 mmk. It seems both maximums sufficiently closely correspond to those observed in the cells. However, the proofs quoted by these authors for the participation of ubiquinone in the cell luminescence are quite problematical and essentially may be summed up in the finding that treatment of the cells with chloric acid in soft regime is accompanied by marked weakening of luminescence. Continuing the treatment with the same reagent in harder regime, these authors found that luminescence again flares up. These waves of luminescence were interpreted by the authors as follows: chloric acid ias a weak oxidizer transforms ubiquinones into the oxidized form and cell luminescence is extinguished, since protein in it does not luminescence because of the strong screening by the oxidized ubiquinone and nucleinic acids. But with further removal of the nucleinic acids and ubiquinone with chlorid acid, the screening effect if removed and the protein begins to fluoresce.

It is not difficult to see that such an explanation is not acceptable, were simply for the reason that, based on the protein content inthe cell, of nuceinic acids and ubiquinone and their molar extinction, it is possible to calculate that the share of protein would be on the average mover 50-70% absorbed quanta. Even in the viruses, rich in nucleinic acids, $^{2}/_{3}$ of absorption at 280 mmk. is the share of protein (Daddy, 1957). And in the case of mitochondria, containing

65-70% protein, 25#30% lipids, 0,5% ribonucleic acid and only 10^{-2} % ubiquinone, the protein absorption portion in the region 250-300 mmk. is about 90% quanta. Moreover, from the point of view of luminescence microscopy, the cell may be considered in the first approach as an aqueous solution, containing on the average 8,5% or protein and 1,5% nucleinic acids and having an optical thickness of 10 mmk (0,001 cm.). Taking the average optical density of the 1% protein solution in the region of 250#300 mmk as being equal to 5, and for the nucleonic acids as 50, we obtain the optical density of the cell $D_{K1} = {}_1c_1l + {}_2c_2l =$ $= 5 \cdot 8,5 \cdot 0,001 + 50 \cdot 1,5 \cdot 0.001 = 0,150$ For such optically transparent systems screening of some molecules by others practically does not take place. In particular, in the given case, complete removal (elimination) of nucleic acids would result in increased luminescece only by 7,5%. Consequently, there generally just isn't any question of any at all significant screening effect on the side of the nucleinic acids or ubiquinone, that would cause extinction of cellular luminescence.

In their last work, Shudel', Chernogriadskaia, VBrunberg, Rozanov and E. Brumberg, 1964, obtaining, unquestionally, some interesting data on the influence by the respiration inhibitors - pytassium cyanide, sodium amythal, didnitrophenol - upon the cells of ascites of Ehrlich carcinoma; nevertheless still further complicated the question of the elementary nature of the cell under study, including in their number tocopherol and vitamin B_{12}. At any rate, by now there has been formulated sev eral different points of view as to the nature of substances, responsible for the ultra violet luminescence of the cells and its elements. Among the responsible agents, there were

found in the nuclei, the luminescent form of DN acid and proteins, in the mitochondria and cytoplasm, proteins, RN acid, free nucleotids, including diphosphopyridinycleotid, the reconstructed form of ubiquinon, tocopherol, vitamin B_{12}. A more exact qualitative and quantotative picture of the mechanism of ultra violet luminescence of cells may be obtained, apparently, with two methods: removal from the celll of the luminescent molecules and their identification and attracting the maximum possible number of spectral characteristics of primary luminescence in the intact cell which would permit to establish the chemical nature of the carriers of luminescence.

The first question which is capable of clearing up the indefiniteness, should be as follows: is it one or several different molecular centers which create the ultra violet luminescence in the cell?

It is possible to get an answer to this very important question directly in the native, intAct cell, studying its fluorescence spectra. At first glance it seem that already the very fact that the fluorescence spectra of various cells and their basic elements - mitchondria and nuclei - represents a non structured single band, is a proof of the presence of only one kind of fluorescent molecules in the cesll (Fig. 51). But actually in reality, this is not quite so. A single non structured band of fluorescence may, in principle, represent encircling total of closely situated fluorescence spectra of several different molecular centers.

The unequvocal answer answer is possible only after comparison of the family of spectra of fluorescence of cells, obtained with monochromatic stimulation with various wave lengths. At the same time,

in this case of single-center mechanism of luminescence, all the spectra should be identical; in the case of multi-center mechanism of luminescence the contribution (share) of various molecules to the total fluorescence spectrum will change with transfer from one length of stimulation to another and, as a result of this, the fluorescence spectrum of the cell will be changed.+ (+footnote: If there is no energy migration with 100% effectiveness effectivity).

Fig 51 Fluorescence spectra at room temperature.
1 - aqueous solution of tryptophane (concentration 10^{-4} M/l' 2 - mitochondria in physiological solution; 3 - nuclei in physiological solution.

It was found that the fluorescence spectra of a number of objects of microbic and animal or gin - yeast cells and E. coli cells, cells of esophagus, muscles, lung and brain of frog and mitochondria and liver nuclei of white rat - did not depend upon the wave length of the stimulating light within the limits of 230 - 300 mmk. (Fig. 52). These experiments prove that in the formation of the spectrum there participates only one kind of molecules. The same conclusion is drawn from the fact of the independence of phsophorescence spectrum in biological objects from the wave length of stimulation in the interval of 250 - 296 mmk. It is characteristical that even nuclei, rich in DN acid, have at their disposal only one kind of luminescent molecules - their fluorescence spectra and even their phosphorescence spectra at the temperature of liquid nitrogen do not depend upon the wave length of the stimulating light.

Then what is the nature of this single center of luminescence? A reliable answer to this question may be gotten by studying the combination of various spectral and luminescence characteristics of the cell.

Fig. 53 represents spectra of fluorescence stimulation of nuclei and mitochondria of white rat lever. These spectra are practically identical with the protein stimulation spectra. In the stimulation spectra, there are clearly manifested both the long wave - 280 mmk., and the short wave - 227-230 mmk., maximums of absorption of the aromatic aminoacids in ptorein. In the region of wave length greater than 300 mmk., the stimulation luminescence is weak. Similar stimulation spectra have also yeast cells and those of E.coli.

Fig. 52. a - 1,2,3 - fluorescence spectra of fraction of nuclei in saccharose at room temperature with stimulation respectively 265, 280 and 302 mmk; 4- polarization fluorescence spectrum of nuclei after emission, stimulation 265 mmk;
b - 1,2,3,4,5 - fluorescence spectra of fraction of mitochondria in saccharose at room temperature with stimulation respectively 265, 280, 296, 254, 302 mmk. ; 6 - polarization spectrum og fluorescence in mitochondria after emission, stimulation 265 mmk.

Fig . 53. Spectra of effect of fluorescence of mitochondria (1) and nuclei (2) in physiological solution at room temperature.

The appearance, in the stimulation of fluorescence spectra of cells of both bands of absorption of protein of tryptophane makes it incontestable that the preimary luminescence of the cells has a protein nature. In an indirect way, in favor of the protein nature of the centers of luminescence are also the rates of the quanta outputs offluorescence (Konev, Lyskova, Bobrovich, 1963). The high rates of the quanta outputs of mitochondria of rat liver and the cells of

the majority of frog tissues (5-25%) indicate that the center of luminescence cannot be a substance, represented in the cell in microquantities, for instance, coferments or vitamins, inasmuch as their share is hundredths or even thousandths parts of the total number of the quanta of the absorbed cells. The high rates of the output show that the basic substance of the cell is luminescent, the part which absorbs light, i.e. the protein (Table 17). Along with the stimulation spectra, the polarization spectra of fluorescence indicate the protein, tryptophane nature of the primary luminescence of the cells (Fig. 54). In the polarization spectra of luorescence after absorption, as well as in the tryptophane containing proteins, one can see the maximum at 265 - 270 mmk., a gap in the region of 280 - 285 mmk., considerable growth in the dgree of polarization in the long wave region 296-300 mmk., The absolute rates of the degree of polarization also coincide with those of protein - 9 ÷ 14% for maximum at 270 mmk. This excludes the variation with energy migration: a certain non protein center is luminescent, it collects energy from the protein molecules as the result of transfer. Actually, fluorescence, after transfer of energy, should be completely depolarized; in this case, protein form of the polarization spectrum is impossible likewise. At the same time, the polarization spectra clearly demonstrate the participation of both oscillators of the long wave band of tryptophane absorption ^1L_a ans ^1L_b in absorption and radiation of light.

Fig. 54. Polarization spectra of fluorescence after absorption in (1) mitochondria and nuclei (2) in physiological solution at room temperature

Table 17

Quantum Outputs of Fluorescence in Various Tissues of Frog

Specimen	Quantum output, lambda of stimul, 265 mmk %			Qunat. outp. lambda of stimul. 296 mmk %
Muscle (calf of leg)	15	17	15	25
		7.3	6.9	
Tongue	7.3	5.9		23
Esophag. epithelium	8.2		8.0	16
	5.2	4.2	5.8	18
Liver	5.9	4.2	5.1	17
Lungs				
Brain (hemispheres)	9.0			15
Heart	5.4			
	immediately as prepared	after 30 min. 8 M urea effect	10 times refrozen	immediately as prepared

DEfinitely protein form also have the polarization spectra of mitochondria and nuclei after radiaztion (Fig. 52). For the fluorescence spectra there is characteristical the constant and rather low rate of the degree of polarization (about 10%) for the entire long wave profile of the band, elevation of the degree of polarization to 13-14% in the short wave portion of the spectrum. Precisely such a polarization spectrum of fluorescence after emission is characteristical for proteins rich in tryptophane in which the inter-tryptophane energy migration takes place with the participation of electron level L_a^1. The constancy of the rates of the degree of polarization of fluorescence within the limits of 310-400 mmk. and independence of the fluorescence spectra from the lambda of stimulation indicate the presence of only one center of luminescence; the oscillator of luminescence at all points of the spectrum is oriented in space at the one and the same angle to the pscillator

of absorption. Especially clear-cut is the tryptophane nature of luminescence as expressed in the spectra of low temperature luminescence and mitochondria. Even a cursory glance at Fig. 55 and 56 is sufficient to become convinced that the typical tryptophane trident (which it is hard to confuse with anything else) is graphically manifested in the spectra of low temperature luminescence. This trident along with the arm at 480 mmk., is characteristical both for the native nuclei anf mitochondria, and for the various tissues. The degree of polarization of phosphorescence in the spectrum of emission is constant and it has a small negative value of the order of -5.0%. All this exclude the participation of any other centers, beside the tryptophane, in the low temperature luminescence.

The elimination from the cells, first of the free nucleotids and the RN acid (treatment with mitochondria and the nuclei with 10% chloric acid during 18 hrs. at room temperature), and then followed by Dn acid (treatment with hot 5% chloric acid during 40 min.) does not produce any marked changes in the above enumerated spectral characteristics of luminescence. This, once again, confirms the exclusively protein (tryptophane) nature of cellular luminescence. The results of experiments with chloric acid, obtained in our laboratory, contradict the results, obtained with the aid of microscopical technique in the laboratory of E.M Brumberg. According to the data of this laboratory, gradual removal of the free nucleitids, RN acid and DN acid causes more and more progressive weakening of the fluorescence; from the data of most recent investigation, fluorescence if extinguished first, and with harder treatment of the cells with chloric acid it flares up. Apparently, the cause of discrepancies in the results may be sought in the fact

in the fact that in the case of luminescence microscopy, a number of secondary factors influence the intensity of luminescence , and first of all the partial elimination of tryptophane and changes in the volume of the cell, as mentioned by Agroskin and Barskii, 1961. But the main cause of the "play" in the intensity of luminescence of the cells, registered by the luminescence microscopy method, consists still in the fact that the chloric acid sharply elevated the tryptophane capacity for photochemical oxidation. While working with the suspensions of cells in macroscopical variation of stimulating fluorescence, the light is greatly weakened during passage through the monochromator. But in the ultra violet luminescence microscope very powerful streams of light are focused on the cell which promotes photodestruction of tryptophane in the cell. Speeding up photo-oxidation of thyptophane in the free state with chloric acid, and also in the composition of the protein or nuclei was registered during radiation of specimens in the focus of the lamp SVD-120A.

Fig 55 Phosphorescence spectra of nuclei in physiological solution at the temperature of liquid nitrogen depending upon the wave length of the stimulating light. Wave length in stimulation, mmk: 1 -265; 2 - 270; 3 - 280; 4-292; 5 - 296; 6 - 302.

Fig. 56. Phosphorescence spectra of mitochondria in physiological solution at temperature of liquid nitrogen depending upon the wave length of stimulating light. Stimulation wave length, mmk.: 1 - 265; 2 - 270; 3 - 280; 4 - 289; 5 - 292; 6 - 296; 7 - 302; 8 - 313./

For example, the quantum output of fluorescence of nuclei drops from 2 to 1% after treatment with hot chloric acid, and after one

minute radiation in the focus with lamp SVD-120A still another six times (Table 18).

Table 18

Quantum Outputs of Fluorescence in Cellular Elements

Specimen and condition	Quantum Output 280 mμk	265 mμ
Tryptophane in distilled water	0.22	0.22
Tryptophane in distillдd water after radiation in focus, SVD-lamp 120A① during 1,2,3 and 4 min.	0.22; 0,16 0.16;0.16	0.22; 0.16 0.16; 0.16
Tryptophane after effect and radiation in focus, lamp SVD-120A during 1,2,3 and 4 min.	0.18' 0,12 0.09 -	0.18; 0,11 0.09; 0.09
Tryptophane in mixture with mitochondria	0.30 -	0.30 -
Native nuclei in physiological solution	0.02 ÷ 0.03 0.01 ÷ 0.015	0.01 ÷ 0.02 0.008 ÷ 0.015
Treated nuclei		
Nuclei without DN acid after 1 min. radiation SVD-120 A	0.0015÷0.0025	-
Mitochongrine in phsiol. solu.	0.1÷0.15	-
Mitochondrine in 8 M urea	0.11÷0.17	-
Mitochondrine after boiling	0.11 ÷ 0.17	

① Radiation was done in a closed cuvette under conditions inhibiting income of oxygen from the air, in the layer of complete absorption.

The photochemical disintegration of tryptophane and proteins in the absence of chloric acid occurs much slower.

In this manner, from the viewpoint of the protein nature of cell luminescence and its elements, essentially, there only remains to explain two closely related circumstances, mentioned by Brumberg and also in the very beginning of this division (chapter)/

First of all, why it is possible not to observe a direct connection between the intensity of luminescence and tryptophane content in the cell, and why, in the second place, there are possible wide variations of intensity of this luminescence? The intensity of cell fluorescence is found to be non proportional to the amouny of tryptophane contained in it, because of several different reasons:

changes in the ratio of active an inactive absorption, i.e. differences in the degree of screening of tryptophane by other molecules (for example, nucleinic acids, hemoglobin, etc.), the light disseminating properties of the cell mediu, the actual assortment of individual prote ns in the given cell, each of which has its own greatly variable quantum output, and, finally, and this is quite important, the character of the supreamolecular packing of the protein which, as this has been shown earlie in case of milk casein, is capable of changing the quantum output of fluorescence more than twice. The combined, occasionally, variously directed of all these four factors must to a certain degree disrupt the proportional ratio between the intensity of fluorescence and the tryptophane content in the cell, although as a whole, the positive correlation, undoubtedly, must be manifested. In favor of the latter are for instance data of Brumberg, Meisel and others, 1958; they observed marked flare up of luminescence in yeast cells after these were fed some tryptophane. All these factors permit the tryptophane luminescence of the cells as though to "feel" the functional physiological or pathological changes in the cells and the organism.

In this manner, the entire combination of experimental data shows the protein nature of ultra violet luminescence of cells. At the same time, the hypothesis on the nucleid nature of the luminescence even with the correction of the existence of two physico-chemical forms of nucleinic acids - the luminescent And the non luminescent (Brumberg, Barskii, 1960) - is not confirmed experimentally. The only instance, mentioned by these authors in favor of the existence of a luminescent form of nucleinic acids - is that of the large

chromosomes in the cells of the salivary glands of the larva of Chironomus. In this specimen, there was observed pronounced fluorescence of chromosome discs, rich in DN acid and, conversely, weak fluorescence in the inter-disc areas of the chromosomes, essentially containing protein. But even this example evokes certain doubts. First of all, this observation, in any case, offers no basis for speaking about life-time existence of luminescent form of DN acid, inasmuch as luminescence was registered in the cells of salivary gland, fixated in Carnois (spelling?) solution. Under these conditions, the appearance of artifacts, associated with the formation of luminescent products of chemically altered chromosome material. Moreover, the chromosome discs gave greater, than the interdisc areas, intensity in the blackening of the photoplate also in the penetrating light 250-280 mmk., i.e. possessed greater absorption. Under the conditions of absence of any qualitative characteristics ot light absorption and the intensity of luminescence considerably greater intensity of luminescence of the chromosome discs could be successfully be produced simply by much greater absolute content of protein in them; this could be felt, in spite of screening from the side of the nucleinic acids. At any rate, the question of the luminescence of the given specimen is not clear and deserves more careful study.

Apparently, all animal and microbe cells possess one, common universal mechanism of ultra violet luminescence - in all cases, the luminescence is conditioned by singlet electron-stimulated states of tryptophane in protein. Somewhat different is the state

of affairs in the case of plant cells, rich in cellulose. It was shown (Konev, Bobrovich, Lyskova, 1964) that in the cells of the root system of the cereal plants, the protein maximum of fluorescence spectra at 340 mmk. appears in pure form only with the monochromatic stimulation with light 280 and 220 mmk., i.e. with selective stimulation in the maximums of absorption of aromatic aminoacids of protein. For the remaining wave lengths of stimulation, the protein luminescence in a greater or lesser degree was camouflaged ("masked") by a wide, washed-out band with a maximum at 430 mmk. This fluorescence had a wide region of stimulation - from 230 to 365 mmk. - and it is cpnditioned, in most probability, by cellulose. Therefore, in the case of plant cells, rich in cellulose, we are dealing, in contrast to the animal and the majority of the microbic cells, not with one, but with two centers of luminescence, responsible for the ultra violet luminescence.

The stage following after establishing of the protein nature, in the study of the ultra violet luminescence of the cell - is the question of the physico-chemical, energy state of the protein tryptophane, in its natural life-term environment. Taking into consideration the high sensitivity of tryptophane to the properties of the medium, the microsurroundings, one would a priori assume that the tryptophane, to a certain degree, may fill out the role of a peculiar would-be index lamp, reflecting perturbations in the colecular organization of the cell. Thus, what influence then on the luminescence has the inclusion of tryptophane residues along with the protein-"carrier" in the composition of the biological structures? And first of all are there manifested in the xellular proteins and its struc-

tures two singlet electron-stimulated states of protein tryptophane - this being one of the most characteristical peculiarities of the energetics of this aromatic aminoacid?

As already mentioned above, the most reliable proof of the existence of one or two singlet electron-stimulated states of proteins in the cell may be obtained while studying the polarizational spectrum of fluorescence of the cell after emission. From Fig. 52, it may be seen that fluorescence of mitochondria and nuclei has constant values of the degree of polarization in the middle and in the long wave portion of the bnad. At the same time, with approaching of the short wave margin of the band of fluorescence there is found quite distinct increase in the degree of xxxxxxxxxx polarization: while the nuclei and mitochondria in the main portion of the fluorescence spectrum have a degree of polarization equal respectively 9 and 8%, then in the region of 300 mmk. these values increase to 11-12%. Similar polariaational spectra after emission have been also received for the suspension of yeast cells. Inasmuch as the character of polarization spectra of fluorescence after emission does not change at all perceptibly after fixation of the cell, the relative probable radiations from the electron levels ^1L_a and ^1L_b do not depend upon the structural organization of the cell or any peculiarities in metabolism in it. Generally speaking, just like the inclusion of tryptophane residues into the protein composition, further inclusion of protein proper into the supramolecular giological systems has little effect on the polariaational spectra. Therefore, the number and the mutual orientation of the main oscillators of absorption, fluorescence and phosphorescence in the cell remain the same, as in the tryptophane residues in free state. Slight variations are

observed only in the relative intensities of the oscillatory (vibratory) sublevel at lambda 285 mmk. of the electron level ^1L_b. It is typical that for the total fund of the protein molecules nuclei this sublevel has xxxxxxx somewhat greater intensity yhat for the total fund (stock) of protein molecules in the mitochondria which is manifested in the existence of a secon minimum of polarization spectra. Already this very fact shows that the physico-chemical energy state of tryptophane, in the composition of various biological systems, may be different. The energy identity of tryptophane nuclei and mitochondria is markedly manifested in the position of the band of fluorescence. In the freshly isolated specimens of mitochondria the position of maximum of fluorescence varies in different animals within the limits of 335-350 mmk. Contrary to the mitochondria, the nuclei have unchanged, stable position of maximum at 333 mmk. In other words, the fluorescence spectrum of nuclear tryptophane is shifted into the short wave side, asd compared to the fluorescence spectru, of mitochonrdia. This means that the tryptophane residues in the nuclei are surrounded by a less polar medium than in the mitochondria.

Likewise, the position os fluorescence maximums of various frog tissues markedly differ ont from the other. In spite of the fact that in all of the tissue, the same molecules luminesce, from the chemical point of view, - still the tryptophane residues - the data of Table 19 show that the fluorescence spectra have a certain tissue (organ) specificity. At the same time, for the given ps species tissues of organs there is its own peculiar, little varying from one individual to another, position of fluorescence maximum. The shortest

shorest wave fluorescence maximum is observed in esophageal epithelium, and the longest wave maximum in the cells of the tongue.

Table 19

Position of Luminescence Maximums and their Relative Intensity in the Tissues of Frogs and Certain Microorganisms

Specimen	Lambda maximum fluorescence, mmk				S/T ratio
	native state t=20°C	8M urea t=20°C	water extract t=20°C	native state t= -196°C	
Tongue	330, 330, 330 [1]	340	340	320	2,35; 2,9
Lung	323,323,323	340	340	319	3,1;3,2;3,36
heart	321,320,322	340	342	319	3,2;3,0;3,1
Brain	324,324,324	340	340	319	2,6,2,57
Esophagus	320,320,320	340	340	319	3,0;3,5;2,5
Liver	328,328,328	340	350	320	2,35'2,35' 2,30;2,20
Muscle, calf of leg	325,325,325	340	340	321	1,8;2,1'1,8
Yeast cell suspension	324, 324			320	1,55;1,40
E.coli cells suspension	340			331	1,75 1,0

[1] Values are given from 3 different individuals.

The intertissular differences in the tryptophane state may be seen also by comparing the relative intensity of the singlet and triplet luminescence. From Table 19 one can see that the ratio of intensity of fluorescence and that of phosphorescence in their maximums (S/T) varies considerably from one tissue cells to another tissue cells.

Consequently, the "average" tryptophane residue in the cells of different tissues is in an environment, constantly changing in its polarity and the degree of association (bond) of hydrogen in the amin group. AGAINΔ THIS MAY BE a result of two different causes: either differences in the primary

structure of the macromolecules of protein, i.e. differences in the composition of proteins, and their assortment, or else differences in the secondary, tertiary or quaternary structures.

The structural character of the cause, responsible for the differences in the position of the fluorescence maximums, is particularly graphically demonstrated in the experiments with the effect of the 8 M urea on the various tissues. As we know, urea does not produce any changes in the fluorescence spectrum of tryptophane proper and at the same time is capable of chAning the position of maximums of protein dI fluorescence by means of disturbing the seoondary and tertiary structures during denaturation. At the same time, the position of the fluorescence maximums of various proteins is stabilized at 350 mmk. This circumstance permits to use 8 M urea was would-be "developing agent" for the nature of the factor, responsible for the intertissular variation in the position of the bands of fluorescence. As one can see from the Table, keeping various tissue in 8 M urea during 30 min. produces a shift int. the long wave side in the fluorescence spectra, not equal in distance. As a result of this, the positions of the maximums of fluorescences of various tissues become unified at 340 mmk for heart, brain, lungs, esophagus, skeletal musces, as well as liver.

Hence, precisely the differences in the rpt ein structures are the immediate cause for the intertissular differences in the fluorescence spectra.

However, this experiments does not permit us to define the two above mentioned potentialities - differences in the average assortment of prp eins or differences in the supra molecular structure of the same proteins, inasmuch as urea is capable not alone to dena

denaturate proteins, but also to destroy the supramolecular structures, appearing at the expense of hydrogen bonds or some other weak forces. To make a choice (selection) between these potentialities is possible by comparing fluorescence spectra of the stock of protein molecules of cells of various tissues in the free state, in solution. If the proteins of various tissues in free state do preserve spectral diffrences, then we are dealing with the first caase - that is the variable protein composition of different cells. But if the free proteins of cells of various tissues give identical fluorescence spectra, this would indicate that the formerly observed differences were a consequence of the different nature of packing of proteins in the supramolecular associates.

The study of the fluorescence spectra of water-salt extracts pf various organs has shown that in this case the differences in the position of the fluorescence bands practically disappear. For these tissues, th re is observed a maximum at 340 mmk., with the exception of the liver, the maximum of which proves to be shifted to 350 mmk. Therefore, the total stock of protein molecules in the cells of the various tissues and organs does not differ in its composition (from the standpoint of luminescence method), but is at different state of supramolecular structure formation.

Cell Luminescence and Its Functional State

Studies on the primary ultra violet luminescence of cell would have very slight practical or theoretical significance for biology, were there not established three groups of facts: 1) that the cells of various tissues differ by their luminescence; 2) that luminescence changes with changes in the functional physiological state of the cell;

3) that the changes of the cell int pathological state are accompanied by corresponding changes in luminescence.

Let us consider briefly some facts that confirm this.

1. Back in the works of Barskii, Brumberg, Bukhman, Vasil'evsakaia and Pluzhnikova, 1959, it was observed that in the germ of hyacinth, the meristematic tissue luminesces in the region of 340 - 380 mmk. more than do the cells of the ovule (ssed bud). An intensive luminescence was also noted in the meristematic tissues of other botanical specimens

Great differences in the intensity of luminescence are characteristical also for the cells of white blood (Brumberg, BArskii, Kondrat'eva, Chernogriadskaia, Schudel', 1960). The most fluorescent is the cytoplasm of the myeloblasts, lyelocytes and metamyelocytes. The rod stab-nuclear and segmented neutrophils fluorescence less intensively. The lymphocytes of the peripheral blood and bone marrow fluoresced little.

According to the data of Khrushchev, 1964, in the connective tissue there is strong luminescence of hystiocytes, mast cells, leukocytes andß and non differentiated (cambiogenetic) cells. The fibrous structures of the loose not formed connective tissue, as well as the cells of the ß fibroblastic series fluoresce poorly.

The changes in the intensity of lfuorescence were observed in our laborztory for the different tissues of frog. The greatest intensity of fluorescence was found to be characteristical for the transversely striated muscle tissue. If we takes its intensity as a unit, then luminescence of cerebral hemispheres will be 0,6m that of the epithelial cells of the esophagys 0.53, tongue 0.55, lungs 0,4, luver 0,30, heart 0, 35. Considerably more interesting than the determina-

tion of the difference in the luminescence intensity of the cells of various organ tissues, is the investigation of its spectra. Assuming that the nature of the primary luminescence of the cell is exclusively protein, it is possible to associate changes in the intensity of the luminescence of cells of various tissues with the variations in the ratio of the "ctive (protein) and inactive (other substances and first of all nucelinic acids) absorption. Then the differences in the spectra of fluorescence of the cells, conditioned by one and the same center - tryptophane - may be associated only with the fact that the tryptophane residues are in not identical physicochemical state.

2. The very first measurement of the fluorescence spectra (Brumberg2, Meisel' and others, 1958; phtographic method in ultra violet luminescence microscope) showed the age ratio in the fluorescence spectra of the tissues. In the adult mice, the collagen fibers of the transverse section of the tail had intense short wave luminescence with maximum about 313 mmk., conditioned, apparently, ty tyrosine of proteins which on more exact determination was found to have a maximum 304 mmk. At the same time, in a young mouse, the collagen fibers did not give such fluorescence. These authors justly associate these differences in the spectra with the age changes of the chemical composition of the tissues. In other observations under luminescence microscope, there was see extinction of fluorescence of cells of rat bone marrow, while they withered away in physiological solution. Much more extensive (Brumberg, Barskii, Kondrat'eva, Chernogriadskaia, Schudel', 1960). Unequivocal interpretation of this observation is very difficult, since there is possible a trivial mechanism of passage of the cellular substance (protein) into the physiological solution

as it dies away. Much more interesting are the observations of Pil'shchik and Nikolaeva, 1962, relative to the age changes in the intensity of fluorescence of the hepatic ells in the white rat. In a whole series of repeated observations, they succeeded in recording a regular course of changes in intensity of fluorescence of the cells, during the process of embryogenesis: weak intensity of fluorescence in the cells on the 14-15th day of development, was followed by a certain increase by the 16th dat. The first wave of fluorescence, then, was replaced by a weakenin by the 17-18th day and by a second wave of flare-up of fluorescence on the 19-20th day. On the 21st day, of the embryogenesis and on the first day of established development the intensity of fluorescence again considerably diminished. These data indicate some regular qualitative changes in the cells during the development of the embryo on the molecular level. Bresler et al 1964 came to make a more unequivocal interpretation of the nature of the periodical chAnges in the intensity of luminescence of the liver cells. In their epxeriments, they studied the dynamics of changes in number of luminescence-microscopical and cytological characteristics of hepatic cells of rat during the process of regeneration, occurring after the removal of about 65% of liver tissue on the rat. At the same time, the intensity of the ultra violet luminescence, during the course of the process of regeneration, changes similarly, as in the case of the embyogenesis. These authors came to the conclusion that the "changes in the intensity of the UV fluorescence of the cells in the regenerating liver are associated with the changes in the quantity and the structure of its proteins."

In the same group we might clAssify the data of L.M.Rosanova,

I. Ia. Barskii and V.M.Brumberg, 1963, - observations on the age physiology; these concerned with more intensive fluorescence of lymphoblasts from the lymphnodes than in the mature lymphocytes of the peripheral blood, as well as erythroblasts (little intensity), as compared to the erythrocytes which do not fluoresce at all.

This latter fact can be quite naturally explained, if we consider that erythrocytes consist predominantly of hemoglobin, in which tryptophane fluorescence of globine is extinguished as a result of migration of energy, absorbed by it into the heme (Teale, 1959).

A number of conclusions could be drawn on the relationship of the ultra violet luminescence of the cells and their differentiation during the process of ontogenesis, from the luminescence microscopical observations of Khrushchev (1964). He mentioned that in proportion to the differentiation of fibroblasts in the connective tissue of mice, their fluorescence fades out. In the mature fibroblasts, the greatest intensity of luminescence belong to the perinuclear regions of protoplasm.

By the present time, there was been accumulated vast material, demonstrAging the profound chemical and physico-chemical reconstructions in the cell during its preparation for the process of mitosis and in the various stages of the mitosis proper. A good confirmation of this may be found in the results of studies on the intensity of ultra violet luminescence of cells during mitosis (Brumberg, Meisel', Barskii, Zelenin, Laipunova, 1961). These authors showed, in the example of the interwining line of the cells of human amion, primary cultures of the cells of guinea pig and moneky and the culture of human embryonal epithelium and bi fibroblasts, that weak luminescence of the

amnion and bhe fibroblast cells in yhe interkinesis is intensified in the early prophase and attains its maximum in the middlestage of mitosis - the metaphase. Then later it gradually it becomes extinguished. In the qualitative evaluation of the changes in the intensity of the luminescence, these changes correspond to the changes in the absorption of light at 250-270 mmk. This latter fact brings up the thought that changes in the intensity of fluorescence reflect the flare up of biosynthenitcal processes in protein, containing tryptophane. This is the more probable, since with the aid of macroscopical methods, in the suspension of the synchronized yeast cells, under the conditions of complete absorption, it was impossible to detect any changes in the intensity of fluorescence of the cells during preparation of onset of the state of budding (Konev, Nisenbaum, Lyskova, 1965). It is, apparently, artional to assume that not alone such decisive in the life of the cell events, as/the process of division, are reflected in the luminescence. One might think that it "feels" and both the qualitative and the quantitative changes in metabolism and in energy.

In reality, of late there appeared some isolated individual observations in this direction. E.M.Brumberg, I.Ia.Barskii, NAChernogriadskaia and M.S.Schudel'. 1963, mention the dependence of the intensity of the ultra violet fluorescence of liver cells in adult rat upon the food rations. However, it remains unclear whether such changes may be associated with quantity and quality- quality of proteins.

In his experiment, Konev, 1964, studied the quantum output of fluorescence of a thick smear of mitochondria of rat liver in proportion to

portion fo fasting ("starvation"). There was found a decrease in quantum output by the 6-7th day of fasting by 10-15%, both for the light of 265 mmk and that of 280 mmk., under the conditions of full absorption. Such a parallelism in the decrease of the quantum output shows that the effect is not just an increase in the screening influence of the nucleinic acids, but rather consists in the qualitative changes in the stored up protein molecules.

Khrushchev, 1964, noted differences in the intensity of luminescence in the same cells of white blood - lymphocytes - depending upon the fact, whether they are found in the connective tissue or in the peripheral blood. At the same time, the greatest luminescence intensity was in the cells, situated in the connective tissue. In the given case, there was distinctly demonstrated association between the cell lumines ence and its metabolism. The regular changes in the intensity of fluorescence are observed als in leukocytes during phagocytosis of microbes (I.M.Brumberg, E.M. Brumberg, 1964).

The segmented nuclear leukocytes of the white mice blood, having captured microbes, had on the average 20% greater intensity of luminescence than the non phagocyting cells. At the same time, the main mass of the cytoplasm in the phagocytic leukocytes luminesced approximately with the former intensity and only the regions situated near the microbe being digested in the cytoplasm luminesced quite brightly. Apparently, the most natural and at the same time very interesting explanation of this flare up may be considered to be the wave of conformational changes in the "working" molecules of protein, associated with the increased ferment activity in these regions of the cytoplasm - the proteolysis, oxidation processes, and the like.

It was shown in the epxeriments of Konev and Lyskova, 1965, that addition to the hungry (fasting) mitochondria, placed into the physiological solution during two or several hours and deprived of substrata for respiration, succinic or citric acid, being the natural participants in the cycle of tricarbonic Acids, reults in a gradual drop in the quantum output of fluorescence of the mitochondria (Fig. 57).

Fig. 57. Changes in the relative quantum output of fluorescence (stimulation 265 mmk.) with addition of succinic acid by drops, at room temperature, to 3 ml. of mitochondria suspension. 1 - freshly isolated ("live"); 2 - fixated with heat; 3 - deprived of viability by hunger (24 hrs. in physiological solution); 4 - changes in light dispersion for the curve 1; 5 - the same as in 1 but with addition of citric acid instead of the succinic acid.

No effect is observed either in the case of the mitochondria, deprived of their vital activity through prolonged fasting or heat inactivation. In such cases, the quantum output remains unchanged in proportion to the addition of succinic acid, and this excludes the possibility of direct interéffect between proteins and the succinic acid, as a cuase for the changes in the quantum output. This suggests that succinic acid has a biological effect, through the processes of biological oxidation.

In this connection, we cannot pass by without mentioning the works of Ungar and Roman , 1962, permitting us to formulate a conclusion, exteremely important in molecular biology, that proteins have a level in their structuration during the course of one of the basic physiological processes: conduction of the stimulation along the nerve.

Conduction of nerve impuses along the nerve with the frequency of sequence of 30, 60, 100, 180 sec. was accompanied by revesible decrease of fluorescence intensity by 30%. The form of the spectram of stimulation and the spectra of this luminescnce, as well as the fact that the luminescences centers did not pass into the physiological solution, used for dialysis, from the nerve extract, indicate purepy protein nature of luminescence. It is remarkable that similar drop in intensity of protein fluorescence of the nerves was observed also under the effect of 6 M urea. All this permitted these authors to promote a very attractive hypothesis to the effect that the nerve proteins directly participate in the production of stimulation, undergoing reversible disturbances of secondary and tertiary structures, similar to those in denaturation.

Changes in the protein macromolecules in the stimulated nerve were also demonstrated by a number of authors with method independend of luminescence: associated with the increased number of suffahydrate groups capable of ionization (data of amerometrical tirtration, Ungar and Romano, 1958), associated with ultra violet spectra of protein absorption (Ungar, Aschheim and others, 1957).

Shtrankfel'd (1964 A) attempted to detect changes in protein fluorescence of the muscle at the moment of its functioning. With tetanic contractions of the sartorius muscle on frog, she observed four-fold weakening of the intensity of fluorescence. With heat (thermal) contracture (according to her ddata), the fluorescence intensity at first weakened, and then strengthened, and under the effect of 2 -10% urea it increased twice in value.

Cell LUminescence and the Pathological Process

From the literary data, we can come to the conclusion that luminescence in a number of cases may be quite a sensitive index for pathological state of the cell. The intensity of luminescnce of cells changes perceptibly during their injury with the ultra violet rays or ionizing rAdiation. Brumberg and Barskii, 1960, observed weakening of the ultra violet and the appearance of the blue fluorescence of the cells in various animal tiasue in proportion t their irradiation with intensive streams of ultra violet light ir ronetgen rays. Apparently, the long wave luminescence was conditioned by products of photochemical oxidatation of proteins or other components of the cells. In favor of this speaks, for instance, the decreased effect in the presence of such a strong acceptor of oxygen as hypo-sulfate sulfide.

According to the data, obtained in our laboratory, radiation of tryptophane in the polyvinyl alcohol results in the appearance of some photochemical products with fluorescence maximums at 420 and 440 mmk. Brumberg adn Barskii, Pinto, 1961, Pinto, 1962 discovered quite high sensibility of luminescence in the cell even with relatively small doese of ronetgen rays, with which it is doubtful that there could be demonstrated any changes in the physical and chemicai properties of protein or nucleinic acids. using other methods. In the experiments of Khan-Magometova, Gutkina, Meisel', Agroskin and Korolev, 1960, it was shown that the intensity of luminescence of various tissues and organs of white rat increased already in 3-4 hours after roentgen radiation with a dose of 1000 r. Taking into the account that, as it has been mentioned earlier, in the animal tissues, only

the proteins containing tryptophane luminescenee, it may be assumed that in the given case also, the observed effects reflect changes in the quantum output of tryptophane fluorescence in the proteins, which have undergon certain structural changes. The independence of the coefficient of blood/plasma absorption in the radiated animals, with simultaneous increAse in fluorescence intensity, is also well in agreement with the concept of structural nature of hte observed effects. Therefore, there appears certain foundations for association of the observed (by these authors(individuality of the tissue response to the identical stimulation (1000 r) with difference in the level of structural development. The greatest increase in intensity of fluorescence was observed for bone marrow - 1.5 times in 4 hours and 1,8 times in 24 hours after radiation, whereas for the spleen, the amount of increase was respectively 1,2 and 1,5 times, and for the liver, 1,0 and 1,05 times.

Barenboim, Barskii, Pinto, 1961, discovered physico-chemical changes in the cells with quite small doses - only 42 rad which appeared 0,5 - 1 hour after radiation. The relative lowering of intensity, observed with these doses, in the blood leukocyte fluorescance, with further increase of the dose changes to a flare up. The maximum positive effect is observed with the dose of 336-756 rad.

In this manner, there is sufficient basis to permit us to ~~xxxx~~ assume that in the observed radiological effect, caused by small doses, the changes in the intensity of the ultra violet luminescence reflect reconstructions, occurring at the level of supramolecular protein structures. In favor of the conformational nature of the luminescence changes is also a certain widening of the fluorescence

spectra, mentioned both by Khan-Magometova and Barenboim et al. Similar widening of the fluorescence spectra is observed in conformational reconstructions of protein, produced by denatuarion by heat and urea, both in vitro and in vivo (Konev, Lyskova, Bobrovich, 1963).

Of great interest are the experiments with observation os cell luminescnce and tissue luminescence in such pathological states as cancerogenesis, inflammatory process, psychic disturbances and muscle atrophy, caused by its denervation. The earliest information on the ultra violet luminescence during a pathological process in the organism were obtained in 1958 (Konev). Barines, Konev, Golubeva, Kuchina and Kobrinskaia, 1958, observed changes in the form of the stimulation spectra of fluorescence of blood serum for schizophrenia patients but did not observed such changes in the group of patients with chronic alcoholism. Similar changes in luminescence, as in schizophrenia, occurred also in the state of experimental catatonia in dogs.

Shtrankelfel'd, 1964, discovered two-phase (first increase, then extinction) of the changes of fluorescence intensity in the sartorial muscle of the frog after its deneration. On the hudredth day after the operation the intensity if fluoresceence in the denervated muscle was loweredby 30-35% as compared to the control - non denervated muscle in the other paw.

Brumberg, Barskii, Kondrat'eva, Chernogriadskaia, Shudel', 1960, mentioned that in acute leukosis, there is a sharp intensification of lymphocyte luminescence of the peripheral blood, whereas other forms of malignant growth are accompanied by increase in fluorescence of the segmented nuclear leukocytes. Rozanova, Barskii and Brumberg, 1963, attempted to

attempted to demarcate somewhat the various forms of leukosis. They succeeded in establishing that the chronic lymphadenosis, in its earliest stages and in case of favorable course of disease, is accompanied by insignificant increase in fluorescence of lymphocytes of the peripheral blood. In severe forms of the disease, in cases of marked metaplasia, the intensity of fluorescence is considerably increased. These authors mentioned that the main carrier of luminescence is the cytoplasm, whereas the nuclei are weakly fluorescent. This reflects well the topography of the distribution of proteins and nucleinic acids in the cell, confirming the role of the nucleinic acids, not as a center of luminescence, but on the contrary, as an effective screening agent. IN a number of works of the Brumberg school (Brumberg, Barskii, 1960) there was observed the fact of opposite influence of a malignant process upon the nucleus and cytoplasm luminescence. In case of cervix uteri cancer epithelial cells nuclei are less fluorescence, and the cytoplasm is more fluorescent than normally. After removal of the nucleinic acids, the nuclei of the tumor cells (ascites and solid tumors in animals) are characterized by brighter fluorescence than normal (Barskii, Zelenin, Liapunova, 1962). From all these statements it follows that these research men were looking essentially for a correlation only between the intensity of luminescence and the pathological process. However, much greater number of peoulirities of structural chemical organization of protoplasm, during the course of a pathological process, will be obtained by comparing not just alone the intensity of fluorescence of various cells, but also the extent of the changes in intensity of fluorescence, under the ffect of a number of factors (light, heat,

ultrasound, various substances in different concentrations). These authors have no doubt that that on principle it is possible to make a specific assortment of tests with stable coefficients of changes in parameters of luminescence, thus creating a very specific catalogue of reliable luminescence, by means of assembling corresponding number of such testing effects! especially if among them there will be included such specific reactions of conformationally corresponding macromolecules as antigen-antibody. In this connection, extremely interesting is the work of E and E. Brumbergs, 1964, in which they actually found marked and easily demonstrable property of the peripheral blood in normal organism and the organism in the earliest stages of cAncerogenous process. In this work, they found that the cells in the transplanted tumor LIO-1 on mice, after keeping in the blood plasma of mice with transplanted tumor have by 30% more intensive fluorescence than after the same keeping in the blood plasma of normal animals. At the same time, the differences in the pacacity of the blood of diseased and normal animals to affect the intensity of the luminescence of the tumor cells appeared in the early stages of the of the malignant growth. In other words, in the early stages of the cAncerogenous process in blood plasma, there are certain easily registered chAnges in its properties and there appears a certain chemical X factor in it which is absent in the peripheral blood of the normal invidivuals.

The clinical importance of such discovery, shouldit be confirmed in the future, cannot be overestimated. The authors also report that with a similar test they succeeded in finding differences in the blood of normal and cancerous persons, and the capacity of the blood to

extinguishfluorescence in the Ehrlich ascitic carcinoma on mice was restored after successful surgical inyervention.

A similar method was used in the work of E.M.Brumberg and E.M. Brumberg, 1964; they showed marked differences in the extent of decrease in intensity of fluorescence of the segmented nuclear leukocytes and lymphocytes under the effect of glycolytic poisons - monoiodoacetic acid and sodium fluoride. This test has been successfully used fo prove the existence and to differentiate between the two forms of acute leukosis - the lymphoid and the myeloid types.

The material reported in this chapter gives us a certain hope that the use of a more varied assortment of luminescent parameters, and in particular the quantitative study of the response reaction of cell luminescence upon the felicitously selected material of experimental effects, in the near future may prove to be very useful auxiliary field aid in the realm of molecular and cellular biology, and above all, in practical medicine.

CHAPTER VI

Electron Stimulated States of Biopolimers and Photobiology

In this Chapter we are nit going to swell in detail on the role of electron stimulated states of proteins and nuceleinic acids in the photobiological processes (it has been quite convincingly proven in a great number of works), but shall limit ourselves only to certain remarks of general nature. An analysis of the literature data permits to point ou four main routes for the participation of the electron stimulated states of biopolimers in the photobiological reactions in the cells.

Ouantum

1. Ouant UFL S' of thymine DN acid T' thymine DN acid dimerization disturbances of the synthesis of RN acid and protein.

2. Ouantum UFL → S' protein (aromatic aminoacids) → photochemistry interaction of photochemical products with DN acid.

3. Ouantun UFL → S' protein (aromatic aminoacids) → photochemistry or protein - damage to the cell function.

4. Quantum UFL → S' → (aromatic aminoacids) → migration and chromophore (provitamin D, protochlorophyllid etc.) chromophore photochemistry → biological effect.

The role of the nucleinic acids as acceptor UFL in the cell during the bactericidal and mutagenic effect of UFL has been shown in many works. Just as unquestionable is the role of tryptophane of proteins as acceptor of UFL in the cell. The spectra of the effect with the protein (tryptophane) maximum at 280 mmk. were found, for instance, for the following processes: inhibition of the property to interfere in influenza virus (Powell, Setlow, 1956), hemoglutination (Tamm and Fluke, 1950), increase in the latent period T-1 of the bacterial phage (after Setlow, 1957), increased number (frequency) of mutation of chaetomium fungus (spelling?) (McAuley and others, 1945; Ford, 1947; McAuley and Ford, 1947); Volvox phototaxis Platimonas subcordiformise (Halldal, 1961); inhibition of hatching of the larvae from nematode eggs (Hollaender And others, 1940); inactivation and spores of mushroom (fungus) Ustilago zeae (Landen, 1939); loss of motility and death of parame paramecium (iese and Leighton, 1935); phototropism of ficomycete (Delbrueck, Shropshire, 1960) and coleoptyl (spelling?) of oats (Curry and others, 1956); inhibition in the formation of spindle and the mitotic apparatus in neuroblasts of the

locust (CArlson, 1954); formation of the pale spot in chromosomes in the xxyqmm tryptone cells - destruction of the nucleinic acids (Zirkle, 1957; Zirkle and others, 1960; Brown and Zirkle, 1964).

Other authors observed approximate constancy of the quantum outputs of biological processes in the region of nuclein and protein absorption. This is manifested in two maximums of the stimulation spectra, situated at 260 and 280 mmk. These ratios are observed for the reaction of inhibition of the infectious phagus T-1 and T-2 (Zelle and Hollaender, 1954), xxxxxxxxxxxx virus B megaterium (Frankling and others, 1953), inactivation and induction of virus E coli K-12m where along with the maximum 260 mmk. there is manifested a small arm (shoulder) at 280 mmk (Frankling, 1954).

The photobiological reactions with participation of DN acids work the fastest with the triplet stimulation states of this molecule. According to the calculations of Mantione and Pullman, 1964, precisely in the triplet state, the character of distribution of density of the pi-electrons facilitates the break 5-6 of the double bond of thymine and the dimerization reaction. xxxxxxxxxxx Beucker and Berends, 1961, came to the same conclusion, based on the decrease in quantum output of photochemical reaction of dimerization of thymine under the effect of paramagnetic ions, extinguishing phosphorescence and, therefore△ DECREASING THE STATIONARY CONCENTRATION OF molecules in the triplet state.

In contrast to the nucelinic acids the "albumine: photochemistry, and therefore, also photobiology peoceeds through the singlet stimulation states of tryptophane. In the experiments of Konev and Volotovskii it was shown that the singlet-singlet migration of energy in the system tryptophane-fluorescein, resulting the

extinction of tryptophane fluorescence of protein, is accompanied by proportional decrease in the quantum out=ut of inactivation of the ferment. Contrary to this, the transfer of energy from the triplet level of tryptophane trypsine to chriz idine, resulting in the selective extinction of protein phosphorescence and decrease of its tau (fluorescence then is not extinguished, i.e. the stationary concentration of the molecules in the singlet stimulated state was not changed), is not accompanied by decrease in quantum output of ferment inactivation. IN other words, the photoinactivation of ferment does not depend upon the concentration of the triplet stimulated states of tryptophane.

CHAPTER VII

Electron-Stimulated States of Biopolymers and Darkness Biology

The problem of electron-stimulated states in the darkness biology may be considered from two points of view.

In the first place, do there arise, in the processes of normal darkness vital activity of the cell, any quanta of light, and if so, do they perform any practical g biological purpose, for example, like physical mechanism in the transfer of information inside the cell or between the cells? and, in the second place, do certain biochemical or physicochemical reactions in the molecule presuppose in darkness, the need of population of the energy levels, manifested in the spectroscopic examinati ns? In other words, can we consider any of the stages in activated complexes of molecules as an intermediate link between the beginning and the terminal products of the chemical reAction as a state of electron stimulation?

Let us briefly consider both of these laternatives.

Bioluminescence in yhe Visible and in the Ultra Violet Regions of the Spectrum

The bioluminescence in the visible region of the spectrum ~~is~~ is well known for many system groups of organisms. It was established in the works of Colli and Facchini, 1952, Vladimirov and Litvin et al 1959-60, Tarusov et al, 1960-65, Konev et al, 1961-65, that in the course of any vital processes there takes place the birth of quanta appearance of light. It is true, the intensity of this universal bioluminescence, peculiar to microbe, plant and animal cells, is extremely small - on the order of 10^{4} - 10^{5} quant/sec woth 1 cm^{2} of cells. The luminescence increases in the atmosphere of oxygen, and is weakened by inhibitors of free radicals and again sharply rises after preliminary radiation by ultra violet rays or ionizing radiation (Konev, Troitskii, Katibnikov, 1961, 1964). At the same time, the bioluminescence is stimulated by super-weak doses of ionized radiation (1-10 rad). Then what is the mechanism of the appearance of this luminescence? conditionally, the mechanism of the apperance of bioluminescence may be subdivided into two stages: creation of a large quantum of energy during the chemical process and stimulation by this energy of some energy level in the substance, directly emitting the quantum of bioluminescence. We shall not discuss the first phase, but let us just mention that there apparently is no basis for/sharing the now generally aceepted not point of view (Vasil'ev, 1962, 1964) that the chemical source of energy in the super-weak ~~mm~~ luminescence is the recombination of radicals, in particular the oxide or hydroperoxide. This is in good agreement even with the fact of weakening of bioluminescence in the presence of inhibitor of free radicals, the propylgallate.

We are more interestedin the question what substance is stimulated by this energy and at which level? We assume that a considerable portion of the light sum total of the supraweak luminescences is conditioned extra-
by the chemiphosphorescence of tryptophane in protein. What are the foundations for such a point of view? First of all, we know from the cell =photoluminescence that the game in the race for the light quanta introduced from the outside is always won by the protein typrophane residues. It would be logical to assume that a similar situation also exists for the quenta of energy, arising endogenously, in the course of the vital chemistry ("chemism").

In the second place, from the systematic studies of Vasil'ev, 1962-64, we know that with the introduction of some foreign molecules into the system where chemoluminescence is in progress, there takes place snesibilization of chemoluminescence with trAnsfer of energy to this longer wave acceptor; the foreign molecules being capable of effectivephotoluminescence. Of all the chromophores of the cell which have the greatest concentration (carbohydrates, fats, lipoids, nucleinic acids, proteins), the latter have the highest quantum output of fluorescence and the lowest level of energy.

In the third place, the spectral composition of bioluminescence of the root system of barley, as shown in experiments with absorption light filters, is quite close to chemoluminescence ot tryptophane containing proteins in hydrogen peroxids (Konev, 1964). Therefore, both in the first and in the second instance, the stimulated one is the triplet level of tryptophane.

In this manner, we assume the participation of the trip let levels of tryptophane of proteins in the formation of the universal supra-

(supra) powerful luminescence of the cells.

In contrast to the super-weak (extremely weak) luminescence in the visible region of the spectrum, these very weak luminescences in the ultra violet region of the spectrum even in their method of detection indicated huge biological activity. As we know, the so-called mitogenetic rays, discovered by Gurvich back in 1923, were detected owing to their property to induce cell division. Grossly speaking, during the addition of inductor, for instance, onion roots, to the detector - the suspension of yeast cells placed into a quartz cuvette, - at a distance, i.e. only through a physical factor, there took place an intereffect between the cells of the inductor and the detector, resulting in increased cell division in the latter. This effect disAppeared, if between the inductor and the detector was placed a glass, not transparent for the ultra violet rays. Hence the ohysical factor of the intereffect was identified as the quanta of the ultra violet light.

Later, the fate of the mitogenesis was quite peculiar. Partly, due to the unusual nature of A.G.Gurvich's theoretical conceptions, and to a large degree because of the certain fastidiousness of the biological method of detection of the ultra violet quanta, the mitogenesis was not practically recognized in the cytological and biophysiological literature. Moreover, the vast application of physical detectors for the extra weak luminescence, started since the 50's, - photomultipliers cooled to low temperatures - resulted in registration of bioluminescence only in the visible portion of the spectrum, as mentioned above. The ultra violet region of the spectrum, then, remained as though "empty". In this connectuon, it became necessary ot make a particularly thou-rough study of the detection of bioluminescence in the ultra violet zone

zone of the spectrum, inasmuch as here could be concentrated the biologically active portion of the radiation. however, before starting the technically complex experiments with the detection of extra weak streams of bioluminescence in the ultra violet region of the spectrum, we believed it to be essential to make an artificial model of the mitogenetic luminescence, to become convinced in the possibility of stimulating the process of cell division, as a result of one of several quanta getting into the cell. In our opinion, precisely this circumstance is the most paradoxical one: in the cell, i.e. in the system, consisting of 10^{13} - 10^{14} molecules with many times mutually duplicated functions, we must admit the possibility of changes, involving this entire system as a whole, from the single electron stimulation of only one (or a few) molecules. Therefore, it was necessary to admit a very effective mechanism of strengthening, resulting from one electron stimulared state. At the same time, the possibility of photodimirization of thymine in the only one of the non doubled cell molecule - the molecule of the DN acid (in the eell, in the state of interkinesis, according to the genetic code, i.e. according to the structure of the DN acid molecules, there are no two identical ones), we have to exclude, becAuse the light streams of mitogenetic intensity are capable of inactivating photochemically only one cystron of DN acid from 10^{3} cells.

In our experiments (Konev, Lyskova, 1964) the mitogenetic radiation was "created" artificially, by means of weakening with neutral light filters (metal nets) of the monochromatically isolated areas of the spectrum of hydrogen lamp. In the final analysis, the intensity of the light streams falling on the cell amounted to 10^{4} - 10^{5} quant/sec per

1 cm^2 of the surface of the cell suspension.

We figured out that the decisive proof of the biological activity of the extra weak UV rays may be obtained with the use of the synchronized culture of cells. As we know, all cells in such culture are approximately in the same functional state, in the course of a definite period of time, after the removal of the synchronizing block they have no capacity to divide, and then simultaneously enter into the mitosis phase. As a result of this, during the short period of time the number of cells is doubled up. By the shortening of the latent period of the appearance of the first wave of mitosis, one can judge about the biological activity of single isolated quanta of the ultra violet lught.

In these experiments we used the liquid culture of yeast cells
Ridder, spelling? Torula utilis, synchronized by removing for 3 hrs. from the Ridder medium of ammonia sulfoxide as a source of nitrogen. The cell concentration was 3-6 thous./mm^3. Counting ot the number of cells was done under the microscope in the Goriaev camera.

The radiated and the control suspensions of yeast cells were poured into the quartz rectangular cuvettes from the spectrophotometer SF-4. Both cuvettes were placed into the light-proof camera, attached to the cuvettesection of the spectrophotometer SF-4. The experimental cuvette was continuously radiated with mercury line 280 mmk of the lamp SVD-120A with the above mentioned intensity. The optical breadth of the slit then was 0.05 mmk. The light stream was evenly uniformly focussed over the entire surface of the experimental yeast culture.

Fig. 58 shows the results of one of the experiments with radiation with the synchro

with the synchronized culture of yeast cells by extra weak streams of ultra violet light. One can clearly see the effect of stimulation of the cell divisions, reflected in the sudden shortening of the latent period (on the average 3,1 times). As a rule, as result of the first wave, the percentage of the divided cells in the experiment and in the control coincides. This means that the quanta of the ultra violet light may stimulate only those cells which are potentially ready for division. The results of 14 analogical experiments are represented in Table 20.

Table 20

Changes in the Latent Period (min.) of the First Wave of Division in Synchronized Culture Torula utilis under the effect of extra weak streams of UV light (10^6 quant/cm^2 sec. lambda=28- mmk.)

Latent period, min.		Acceleration of the latent period, number of times
Culture in rhe dark	Culture under the same conditions+UV rays	
135	25	5.4
120	50	2,4
120	30	4,0
100	60	1,7
135	30	4,5
135	40	3,4
150	35	4,3
130	60	2,15
180	45	4,0
100	30	3,3
100	40	2,5
120	40	4,0
80	20	4,0
120	50	2,4
Average arithmetical 123	39,5	3,1

Fig. 58. Changes in concentration of cells in the synchronized culture of Torula itulis in Ridder medium at room temperature, depending upon the period of time of removing the block; continued

Fig. 58 cont.: 1 - in the dark; 2 - with some monochromatic light 280 mmk. Dose 10^6 quant/cm^2 sec.

The absence of transgression in the number of latwnt periods in the experiment and in the control shows that the observed difference is fully reliable and makes it unnecessary to have other variation statistical interpretation of the results.

In the scheme of the experiments for the half-hour period the suspension, containing about 10^7 cells absorbs $10^6 \times 30 \times 60 = 2 \times 10^8$ quanta, i.e. approximately for each cell there is ten quanta throughout the time of the exposure.

Hence, these experiments prove the essential possibility of physical intereffect between the cells, separated by comparatively great distances by means of the UV streams of mitogenetic intensity.

Two more circumstances attract our attention. First of all, the increased strength of the dose up to 10^9 quant.cm^2 sec is accompanied by disappearance of the effect of stimulation; this has been earlier many times observed by the A.G.Gurvich's school. In the second place, with the gradual decrease of the radiated volume of cells (volume of cells being radiated, (diaphragm effect of the parallel bundle of rays, there is observed the effect of the critical mass: the light streams, covering an area over 1 mm^2 prove to be equally effective, whereas the light streams, covering area less than 1 mm^2 (10^5 of cells) are ineffective. This means the valuable mechanism of the transfer of signal to division from cell to cell. At the same time, after reaching the critical mass, the system gave a reaction according to the law "all or nothing", even in the cases when less than 1/100

portion of all cells. It may be assumed that the mechanism of the secondary induction consists of a chain process of the appearance and transfer from cell to cell of a secondary ultra violet bioluminescence, with the aid of which mitosis stimulation takes place.

These experiments confirmed the reality of the attempts to demonstrate the mitogenetic radiation of the cell with the aid of physical detector.

The iltra violet bioluminescence in the original experiments (Troitski Konev, Katibnikov, 1961) was registered with the aid of self-extinguishing gas meter of photones (photons) (of the type of Geiger tube, with a platinum photocatode, sensitive only to the spectral area 200-300 mmk (Shelkov, Prager, Kostin, 1959). The quatz window in the meter has an area about 1 cm^2. The fundus (background) was lowere with a protective lead-aluminum screen and the use of the diagram of anti-coincidences with the protective ring of the meters. The protective ring consisted of 12 pipes of the type STS-6, arranged like an umbrella over the photon meter. To insure better reliability of work of the equipment, the duration of the impulse from the screen meters in the fourth canal (channel "Appletrees" was increased up to several mksec., and the impulses from the meter of photons in 1-2 canalc were held up by a constant line of inhibition for one mksec and narrowed to the fractions of mksec. All this permitted to lower the background from 50-60 to 5-8 impulses a minute. This equipment permitted to register the ultra violet bioluminescence in a number of specimens (Table 21).

Moreover, luminescence of the contracting heart of the frog was registered (elevation above the background 35%, which disappeared at the same time as the heart stopped, and luminescence of crushed frog muscle (20% above the background . In the

intAct muscle, in the state of physiological rest, we could not detect any luminescance. Placing an empty cuvette, instead of the specimen, into immediate vicinity of the quartz window (1-5 mm) or a cuvette with nutritive medium (nutrient) or water did not increase the speed of calculation as compared to the backgournd. The specimens, screened with black paper or thin glass, also showed frequency of count, characteristical for the background. In this manner, luminescence of a whole number of classical "mitogenetic specimens can be registered with thaid of a physical detector. However, the very constancy of the fact fof extrele weak bioluminescence in the ultra violet region of the spectrum still does not prove the auxiliary, informational role of the quanta generated by the cell.

Table 21

Relative Intensity of the Ultra Violet Bioluminescence of Certain Biological Specimens, imp/min.

Specimen (bject)	Average Arthimetical ± square error		
	specimen	background	elevation above the background
24 hour culture of alcoh l yeast of race in grape must wash (wort,	12±1,7	8,3±0,7	47±22
Same in grape must agar	7,2±0,1	5,2± 0,5	42±10
Dandelion raceme, in sunny weather	6.44±0,11	5,35±0,18	20,2±3,9
Same, in overcast weather	5.4±0.18	5.35±0.18	none
Same, after ultra violet irradiation	6.3±0.1	5.35±0.18	17.8±3.6

It would be quite admissible, for instance, to assume that the quanta, appearing as a result of recombination of radicals, simply follow up the processes of vital activity, representing physiological waste refuse products of chemical life. First of all, therefore, it was necessary to clarify whether the process of division of cell is

actually accompanied by emission of quanta of ultra violet light. For the clarification of the ratio between the extremely weak uktra violet bioluminescence and the cell division, we performed experiments with synchronized culture of yeast cells. The synchronization, on the one hand, increased the stationary concentration of cells more than by two orders, the cells simultaneously being in the phase of division, and on the other hand, permits registration of bioluminescence of a great combination of cells, at the identical stages of mitotic cycle.

Fig 59 Synchronized culture of cells of Torula utilis.
1 - changes in concentration of cells in mm^3 of suspension, depending upon the time after removal of block; 2 - ratio of intensity of ultra violet bioluminescence of culture (in absolute predominance over the background in imp/10 sec with background of 6 imp/10 sec) and time after removal of block

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The experiments were carried out as follows. The culture of yeast cells Torula utilis, grown on Ridder medium, was synchronized by removal for 2 hrs. from the nutrient medium of nitrogen salt. After addition of the salt, there followed a two hour latent period, at the end of which there appeared the first wave of budding. Immediately after the removal of the block the yeast culture in the concentration of 10-30 thous. cells in mm^3 was poured into a quartz cuvette and placed in the immediate vicinity of photocatode of the photomultiplier FEU-18A, cooled through the legs of the socle (peddestal) with liquid nitrogen. For the control on the sensitivity of the equipment apparature, we used a luminescent glass ZHS-19, containing small admixtures of a radioactive substance (Uranium). The photomultiplier worked in the regimen of meter of

of meter of photons, the signal was intensified by a wide band impulse intensifier D-4 and passed to the claculating gadget PS-10000. The dark background of the apparatus was on the average 4-8 impulses per 10 sec. with sensitivity of 200-300 imp/10 sec. on calibrating device ZhS-19. The yeast situated in the dark in front of the photocatode, was counted in turn with the photon or boiled culture of the same yeast, as control. The average results of 10 experiments, on the whole giving similar results, are represented in Fig. 59. From this figure one can see that in the very first moments after the removal of the block the culture hardly radiated at all. Approximately in after 20 min. after removal of the block there began marked radiation, attaining its maximum by the 50th minute. Exceeding Elevation above the background in the maximum varied in different experiments in the interval of 20-150% (on the average 70%).

Statistical proof of the excess of speed of calculation above the background or fasting culture of yeast is quite high: t=3,3; this corresponds to over 99% reliability (proof).

Then followed gradual extinction of luminescnce, and by two hours excess over the bAckground completely disappeared. In 2,5-3 hrs. there was outlined the second wave of radiation, corresponding to the second wave of budding. Comparison of the ratios in the curves of intensity of luminescence - time, after removal of the block fxmxxhm with the curves of the increased number of cells shows that the wave of radia# tion got ahead of the wave of budding, evaluated fromthe adult cells, approximately for 1 hour. Therefore, radiation Appears in the yeast cells at the moment when there is preparation and development of the processes of division at the molecular level, not yet appearing in the

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formed morphological elements. In these experiments there is quite apparent the causative connection of bioluminescence with the process of the cell division. The cells, being in the state of interkinesis, - the fasting cells - are deprived of any registered luminescence. The fact that the luminescence is actually in the ultra violet region of the spectrum may be proven in the experiments withthe absorptional light filters. Thus, the light filter BS-6, letting through light (retains) with wave lengths exceeding 380 mmk, almost completely inhibits the mitogenetig radiation.

Eighty (80%) percent of radiated quanta pass through the light filter UFS-2 (250-380 mmk), while the light filter BS-5 (330-800 mmk) passes about 50%. Therefore, radiation represents a wide band between 250-380 mmk with maximum around 330 mmk. It is typical that protein fluorescence likewise is weakened by the light filter BS-5 about 1,7-1,8 times and is situated in the same region.

During the time of the first wave of mitosis, there are registered about 100 impulses. Taking the quantum sensitivity of the photocatode to be 100 quanta per 1 impulse, the body angle as 40°, the factor of screening by the protein and nucleinic acids of the cell luminescence as 1/10, it is possible to evaluate the total number of generated quanta approximately as 10^{8}. Taking into account that in the same system, during this time, there were divided 10^{7} cells, we obtain the fact that each cell during this time of preparation to mitosis radiates about 10 quanta. However, based on the findings, it is difficult to say whether these are radiated simultaneously or whether their radiation (emission) is stretched over a more or less prolonged period of time.

In this manner, these experiments may be considered as the first confirmation, with the aid of physical methods, of the existence of the mitogenetical luminescence (radiation).

Of essential importance is the problem as to which molecules in the cell start, in electron-stimulated state, the chain of processes eventually leading to the biological macroeffect. An answer to this question may be had with the aid of the study of the spectral dependence of the effect of shortening of the latent period in the synchronized culture of cells. For the monolaye monocellular layer it was found that the light with wave length of 280 mmk, directed essentially on the proteins, has 1,5# 2 times lesser threshold intensity than the light of 265 or 254 mmk, directed to a greater extent to the nueleinic acids. This leads us to prefer protein as acceptor of the extra weak bioluminescence of the cells.

This conclusion concurs well with the observation of CArlson et al, with the use of the technique of ultra violet microbundles in the cells of the neuroblasts of locust embryos (1961). Making use of monochromatic micro bundles of 3 mk in diameter, these authors observed inhibition of mitoses with large doses of UV rays. However, with small doses, when the effects of damage have practially no role on the molecular level, there is seen complete reversal of the biological effect: instead of inhibition of mitoses, there is their stimulation. Determining the interval of time, separating the earliest and the middle prophases, on the one hand, and the metaphse, on the other hand, for the "twin" cells of one and the same embryo during identical stages of mitosis, the authors found that of the 12 cells which received a slight additional light 280 mmk in the early prophase, ten (10) reached the metaphase before the controls, and only two (2)

after the control. Of the eight (8) cells which received a weak added light during the middle prophase, seven (7) attained the metaphse before the control and only one at the same time. Such stimulating effect of the speed of the mitosis process fro weak added light 280 mmk was not observed in the case, when the monochromatic line 265 mmk. was used.

In this manner, most likely the protein in the nucleioli has a stimulating effect on the mitoses.

In this connection, we may express an assumption that precisely with the appearance of extra weak ultra violet radioluminescence and corresponding stimulationsin the state of protein is associated the stimulating effect on life vital activity from the small doses of ionizing radiation.

Finally, a few words about the least understood moment in the mechAnism of the effect of single quanta of the UV rays on the cell the mechanism of strengthening of he effect. As a working hypothesis we may assume that in the cell as a whole there are possible certain fine cooperative structural reconstruction of the majority (or of all) protein molecules, starting/with/any photochemically deformed single protein mAcromolecule. The existence of such a structural "phase" cooperative transformation of the entire cell as a whole would at the same time shed some light on the effect of certain chemical substances (colicins), one molecule of which, not interacting with any type of nucleinic acids, is still capable of stopping the protein synthesis at the same time in thousands of cellular ribosomes.

Possible Participation of the Optical Energy Transfers in the Chemical Reactions

Coincidence of the spectra of the photo-, radio-, electro-, and chemoluminescence of the organic molecules, esse tially, has a profound significance; it indicates that, with intereffect of the diffe# rent forms of energy, the organic molecules makes use of the same energy levels, determined by its internal structure. Therefore, precisely the optical levels of energy must use molecules in order to accomplish shifting of their electrons and nuclei which, in the end (final calculation) the electron nature of the chemical reaction. Apparently, in this connection, profound internal unity harmony (oneness) between the photochemical and chemical reactions extends actually much further than we can imagine at the present time. Most probably, it is justifiable also to look not just for the one-sided but also for the two-dided mutual connection; not alone in the result of energy brought in from outside in the form of lightquantum, there may arise various chemical transformations, but in the course of the chemical reactions proper, in its exothermic links may elect as the most effective path for the given reaction, the lectron-stimulated states of the molecules. At the same time, it is not al all indispensable to have the course of such reaction accompanied by luminescence: the constant of speed of the chemical route (path) of deactivation of such molecules may considerably exceed the constant of the speed of optical radiational path of deactivation. Nor is it necessary to overcome the entire energy "distance" between the levels at the expense of some single concentrated source of energy, for instance, recombination of radicals, disintegration of hydrogen

peroxide, etc. This is disproven by the common fire-fly, with its chemoluminescence, in which the energy of the macro-energetic phosphorous bonds, not exceeding 12 kcal, induces stimulated states, corresponding to the energy of 50-60 kcal. As a matter of fact, one of the initial products of the final phase of reaction may contain a great supply of potential energy, and conditions may be fostered in the cell for "collecting" a large quanta of energy from little ones, in sequence, partially, with great effectivity (quantum output of transformation of chemical energy ATF into the light energy about 90%). One of the greatest achievements in this direction may be considered to be the discovery by Vladimirov and L'vova, 1964, of luminescence accompanying the oxidizing phosphorization-in the mitochondria. These authors showed that in the course of this universal terminal process of extracting of energy from the food, there naturally appear some electron-stimulated molecules with a supply of free energy in 60-70 kcal.

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It may be assumed (along with Reade, 1960) that the concrete path of chemical transformations, the nature of the "sympathies" and the "antipathies" of the molecules to some or other types of chemical trAnsformations, as well as in the case of photochemical reactions, to a considerable degree may be predetermined by the energy structure of the molecule, the structure of its energy levels, space anisotropy and the probability of electron transfers (passages). DisturbAnces of the electron density, in this case, may be likened to a control wheel the turn of which predetermin the direction in the movement of a heavy autobus - the atomic nuclei in the composition of the molecule.

The energy distance between the level of the main state of the Substance A and the electron level of the meta-stable interval state of the chemical reaction determines, in this cases, that minimal energy which is required for the reaction - the energy of activation. The direction of the dipole moment of passage (transfer between the energy states of molecule A and the metastable interval product would determine the path (trajectory) of the shifts of the electron from the molecule in complete and partial ionization - stimulations. From this point of view, in each molecule there exist its own selective determined directions of shifting of electrons, with reference to the molecule skeleton which, along with the vector ratios of the supplied energy (for instance, direction of the collision) determine the probability of the accomplishment of the reaction, the steric (spacial) factor in the kinetics of chemical reactions. In recent years, there have been obtained considerable proof of this. We shall limit outselves to a few examples.

At the present time, it is becoming gradually apparent that the processes which are accompanied by transfer of electrons from one molecule to another, are not at all limited to the classical oxidizing reconstructive systems, but rather play considerably more extensive and universal role, determinging the capacity to intereffect of molecules AND MACROMOLECULES IN THE WHOLE circuit of biochemical processes.

The role of tryptophane as donor of electrons is assumed in many complexs of charge transfer which have direct biological significance. Of the greatest interest, from this point of view, is the possible specific combination of ferments with coferments, possessing the pyrimidine ring in the mechanism of complex transfer of charge. In favor of this

is, for example, the appearance of a wide band of absorption during mixing of glyceraldehyd-3-phosphodehydrogenase with nicotine-amide-dinucleotid (Kosower, 1956; Chilento and Cilento and Ginsti, 1959). Also a similar complex is formed with addition of nicotinre-amide-benzochloride to indol or its derivatives; this is manifested in instantaneous appearance of yellow coloration, similar to that observed in the complex of enzyme-conenzyme (Cielnto, Tedeschi, 1961). The appearance of a wide band of absorption with maximum at 325 mmk, belonging to the complex of the transfer of charges N-beta-indolyl-ethyl-3-carboxamide-pyridine-chloride, as shown by Shifrin, 1964, is accompanied by hypochromic effect in the region of absorption of indol proper. In our opinion, that most interesting point in Shifrin'S results is that this decrease of absorption is maximum in those areas of the spectrum, where the oscillator ^1L_a has the greatest relative contribution. In the region of absorption of the oscillator ^1L_b there is observed a marked decrease in hypochromic effect, so that in the maximum at 287 mmk. the differentiational spectrum has the zero values. These results permit us, to Assume that only those electrons may be partially shifted from indol to the pyridine ring during the formation of complex $A^+ B^-$ which participate in the passage at the ^1L_A level. At the same time, the orientation of this biochemically active oscillator with reference to the molecule skeleton and its corresponding spectrum of absorption coincide well with the same characteristics of the usual optical oscillator of indol. Hence, we might propose a quite hypothetical, but important for the dark biology, postulate that the main biological energy process - the process of biological oxidation, is accomplished with the participation of optical oscillators of tryptophane: the oxidized coenzyme is bound

with the tryptophane in the enzyme in the mechanism of transport of charge in the complex of transfer of charge. Apparently, the optical oscillators of tryptophane play a role in considerably greater number of biological processes than we can imagine at the present time. We cannot but feel a(feeling of)surprise over the fact that even before Mulliken, who confirmed citizenship rights and promoted the theory of complexes of transfer of charges, 1950, 1952, the possibility of union of biological molecules with one another, not at the expense of chemical bonds, but by formation of donor-acceptor complexes, has been proposed by the biochemist Brackmann, 1949, for the explanation of the main immunological reaction - the antigen-antibody reaction. This point of view, in recent years, has been supported by Weinbach, 1964. Kanner and Kozlov, 1964. also thus classify the reaction of phage T-2 with indol, and that of phage T-4 with tryptophane; this reaction deprives the phage of the property to epenetrate into the cells of the E.coli. These authors show that for the union with the tail of the phage there is no need in any functional griups in the indol, imidazol, benzimidazol and other types of rings, with the aid of which there could arise a chemical compound. This reaction is in direct ratio only witl with electron-donor properties of inhibitors.

In the given case, the inhibitor appears as the factor which, by means of formation of the complex of transfer of charge with the participation of one of its oscillators, produces structural reconstruction in the protein tail of the phages, thus depriving them of the ability to be attached to the cells of the host microorganism.

In this manner, the optical electron oscillators of the indol ring of tryptophane may play an essential role in the natural biochemical functioning of this molecule, predetermining peculiarities in the participation of the indol ring in such basic reactions, as the fermentative rprocess, and the immunological and mechano-chemical reactions.

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TrAnslated by Tatiana Boldyrefff

Please correct spelling: "chemoluminescence" - thank you.